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TABLE OF CONTENTS ON INSIDE COVER

The Journal of Laboratory and Clinical Medicine

CONTENTS FOR APRIL, 1939

Clinical and Experimental

The Blood Glutathione in Hematologic Diseases. E. V. Kandel, M.D., and G. V. LeRoy, M.D., Chicago, Ill. ---- 669

The "Average Daily Reticulocyte Response" During Therapy in Pernicious Anemia. Arnold Friedman, A.B., Raphael Isaacs, M.A., M.D., and Anne Lufkin, A.B., Ann Arbor, Mich. ---- 677

The Pharmacologic Antagonism of Metrazol and Sodium Amytal as Seen in Human Individuals (Schizophrenic Patients). Louis H. Cohen, M.D., Worcester, Mass. ---- 681

Examination of Effusions in Tumor Cases. Kurt E. Landé, M.D., Hagerstown, Md. ---- 685

The Concentration of Acacia in the Serum, Its Rate of Excretion, and Its Effect on the Colloid Osmotic Pressure Following Intravenous Injection in Cases of Cirrhosis of the Liver. Hugh R. Butt, M.D., and Marschelle H. Power, Ph.D., Rochester, Minn., and Ancel Keys, Ph.D., Minneapolis, Minn. ---- 690

Rapid Conversion of Tryptophan to Indol by Resting Bacterium Coli. Stuart L. Vaughan, M.D., Ph.D., Buffalo, N. Y. ---- 696

Lipid Changes in Nerve Degeneration in Avitaminosis A. Raymond Reiser, A.B., Ph.D., Durham, N. C. ---- 701

Metastatic Bone Carcinomatosis Associated With Parathyroid Hyperplasia and With Symptoms of Hyperparathyroidism. S. Ben-Asher, M.D., Jersey City, N. J. ---- 709

The Reversal of Kidney Volume Response Accompanying Abolition of the Central Nervous System. Edward C. Mason, M.D., Ph.D., and Philip Hitchcock, M.S., Oklahoma City, Okla. ---- 717

A New Apparatus for Oxygen Therapy. Charles F. Lombard, A.B., and Clarence Nelson, M.D., Los Angeles, Calif. ---- 724

Effects of Smoking on Blood Sugar and Peripheral Temperatures of Convalescents. H. B. Cates, M.D., and J. G. Giovannazzi, M.D., Los Angeles, Calif. ---- 729

Laboratory Methods

A Manual of Neurohistologic Technique. Oscar A. Turner, M.D., New Haven, Conn. ---- 735

A Simple Blood Pipette Cleaner. Oscar Bloch, Jr., M.D., Louisville, Ky. ---- 749

Small Animal Holders for Intravenous Injection. K. K. Chen, Ph.D., M.D., and Charles L. Rose, A.B., Indianapolis, Ind. ---- 750

A Simple Method for Visualizing Pressure and Volume Changes Applicable to Observations on the Rhythmic Variations in the Peripheral Circulation. C. Wesley Scull, Ph.D., Abington, Pa. ---- 753

Unreliability of Oxalated Blood in Determination of Erythrocyte Sedimentation Rate. Samuel Berg, M.D., Newark, N. J. ---- 757

Laboratory Methods for Testing Fungicides Used in the Treatment of Epidermophytosis. Ella M. Burlingame and George F. Reddish, St. Louis, Mo. 765

Department of Reviews and Abstracts

Selected Abstracts ---- 773

Book Review ---- 778

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The Journal of Laboratory and Clinical Medicine

VOL. 24 APRIL, 1939 NO. 7

CLINICAL AND EXPERIMENTAL

THE BLOOD GLUTATHIONE IN HEMATOLOGIC DISEASES*

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THE discovery that cysteine-containing proteins are necessary for growth was the basis for some questionable reasoning concerning glutathione. It was assumed by some workers that since cysteine differed from other amino acids chiefly in the possession of a sulphydryl group (–SH), then any compound having this group should display similar properties. In 1921 Sir F. Gowland Hopkins demonstrated that reduced glutathione was responsible for the active reducing properties that some tissues possessed. There has been, since then, not only a great amount of speculation about the role of glutathione; but also there has been sound scientific work. Glutathione, glutaminyl-cysteinyl-aminoacetic acid, is present in all the tissues of the body. Those tissues that are the most cellular contain the greatest amounts. Very little of the substance is present in blood plasma or in the extracellular fluids. The compound exists in the body chiefly as reduced glutathione (GSH). It readily parts with its hydrogen atom (from the sulphydryl radical), and two molecules of GSH join to form "quasi-oxidized" or disulfide glutathione (GSSG). Further oxidation breaks up this compound, and the sulphydryl becomes successively –SO, $-SO_2$, $-SO_3$, and $-SO_4$. The oxidation-reduction system: $2GSH = GSSG + 2H$, is the reaction of the compound of greatest interest to biological chemists.

In a recent study of biological oxidation Lyman and Barron[1] stated:

"It is surprising to find that, although the rôle of glutathione as a respiratory catalyst was put forward as soon as Hopkins discovered it 16 years ago, there is as yet no positive evidence that glutathione functions as such."

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They further remarked that:

"Glutathione and ascorbic acid seem to possess closely related properties in the chemical activities of biological systems, maintaining graded levels of reduction intensity, necessary for the performance of certain biochemical processes. Both substances are present in the living cell mostly in their reduced form, both are catalytically oxidized by copper, and glutathione, like ascorbic acid, is oxidized by atmospheric oxygen with hemin and hemochromogens as catalysts."

In this connection, Hopkins and Morgan[2] have demonstrated that glutathione can prevent the oxidation of reduced ascorbic acid by the hexoxidase of Szent-Györgyi until all the glutathione present is oxidized. Furthermore, oxidized ascorbic acid is slowly reduced by reduced glutathione. In the presence of this hexoxidase, the reduction occurs five times as rapidly. Similarly, reduced glutathione protects ascorbic acid from oxidation by copper catalysis.

Two other observations on the chemical action of glutathione may be mentioned, although they have not been interpreted yet. Tait and King[3] noted that reduced glutathione increased the rate of oxidation of lecithin and other fatty substances. Meldrum and Tarr[4] demonstrated that oxidized glutathione (GSSG) is reduced anaerobically by the Warburg-Christian enzyme-coenzyme system in the presence of hexosemonophosphoric acid.

Contrary to the opinion of Lyman and Barron, Handovsky[5] presented evidence to indicate that glutathione acts as an emergency auxiliary respiratory ferment whenever the oxygen supply to a cell becomes either relatively or absolutely inadequate. His reason for so thinking was that he observed increased amounts of GSH in tissues poisoned with hydrocyanic acid, or exposed to a decreased oxygen tension. This idea, incidentally, tended to support the work of Gabbé[6] who found that in experimental anemia the glutathione quotient $\frac{\text{(GSH in mg. per cent)}}{\text{(R.B.C. in millions)}}$ increased. This occurred when the animals were bled rapidly, or poisoned with phenylhydrazine; and also when they were kept in an environment with a reduced oxygen content.

Of more interest in relation to the hypothesis to be described presently, are two directly contradictory studies. Bumm and Appel[7] reported that reduced glutathione reversibly and specifically inhibited the Pasteur reaction in tissue. (The Pasteur reaction is the inhibition of aerobic glycolysis by respiration, i.e., in the presence of adequate oxygen the conversion of glucose to lactic acid does not occur.) Their data showed that glutathione had no effect on respiration or on anaerobic glycolysis of tumor tissue; but that it increased the aerobic glycolysis to the anaerobic level. If such a conclusion could be verified, it would suggest that glutathione might serve to regulate the Pasteur-Meyerhof cycle in muscle. Then if increased amounts of glutathione could be demonstrated in tumor tissue, this action might explain the break in the Pasteur reaction which occurs in tumors. However, Baker[8] repeated the study and reported that reduced glutathione had no significant

effect on the aerobic glycolysis of tumor, brain, testis, and embryo tissue. She concluded that GSH neither participates in nor affects the Pasteur reaction.

The faulty syllogism which yielded the concept that the –SH radical influenced growth, gave rise to another equally questionable idea. This was, that since cancer is an overabundant growth of tissue, it may be due to a local accumulation of glutathione. Although there is considerable variation in the analyses of neoplasms for glutathione, the majority of workers have not found a significant excess. As a matter of fact, even if such a result were always found, the remark of Hueper[9] in his excellent survey of the significance of sulphydryl as a growth factor would be pertinent. He pointed out that:

> "The mere fact that a certain substance is accumulated in proliferating tissues is, moreover, insufficient proof of its relation to cell multiplication."

The exponents in America of the theory that growth—in particular malignant growth—is somehow related to the sulphydryl radical have been Hammett and Reimann. In general this theory argues that sulphydryl, as the substance occurring naturally in the cell, is the universal and essential stimulus to growth. The reaction product derivatives of –SH are the natural inhibitors of this process, so that the stimulation by –SH is normally limited by the inhibitory action of the suboxidized sulfur forms of –SH. These are $-SO_1$, $-SO_2$, $-SO_3$, resulting from the normal oxidation of sulphydryl. Cell proliferation is, therefore, regulated by the intracellular equilibrium –SH: $-SO_3$. Other mitosis-stimulating agents are said to work through sulphydryl. Experiments of others that failed to demonstrate a disturbance of this equilibrium, and recent genetic studies have led to a slight change in Hammett's[10] views: namely, that the potentiality for malignancy lies in an hereditary determination of lines of cells having (as in the embryo) a heightened sensitivity to sulphydryl. Recently, experiments with carcinogenic hydrocarbons and parathiocresol (which contains the –SH group) have suggested to Reimann and Hall[11] that sulphydryl may exert a protective action against carcinogenesis. How this dilemma will be resolved is not yet clear.

It was inevitable that such a theory of canceration would be examined clinically. Nothing will be gained by reviewing all of the papers bearing on this problem; but two studies should be mentioned. Both of these were performed on blood because it is readily available, and in the hope that changes in it would accurately reflect variations in the composition of tissues with respect to glutathione. Many analyses for glutathione have been made in the blood of patients with cancers. In general the content has been low, and this has been attributed to an absorption or a fixation of glutathione by the malignant tissues. Schoonover[12] examined the blood of untreated and treated cancer patients, and also normal subjects. The average reduced glutathione value for the diseased group was not significantly different than the normal controls. (This coincides with our experience in the case of blood dyscrasias.) She then estimated the ratio GSSG:GSH in the blood plasma, in the erythrocytes, and in the whole blood. In the patients with cancer, treated or not,

this ratio deviated from the normal. How significant this deviation was, is not stated. The plasma and the erythrocyte GSSG:GSH ratios were then divided into each other, giving even larger (numerically) discrepancies between the normal and the cancerous subjects. The significance of these observations is not clear, but it is worth recalling that Gabbé[13] showed that the glutathione quotient (GSH/R.B.C.) usually increased when the erythrocyte count was low. How much influence the common anemia of cancer patients had on the ratios is not apparent.

Parker and Kracke,[14] in the course of an experimental study of agranulocytosis, took up the torch for glutathione as the principal growth-stimulating factor. Their rabbits poisoned with benzol had decreased amounts of glutathione in the bone marrow and the blood. This was taken to indicate that the decreased cellularity of the blood and the marrow was due to a depletion by benzol of the normal acceleration factor for cell division, "which appears to be reduced glutathione." In a series of 14 patients with varied hematologic disorders they observed changes in the glutathione content of the blood. In 3 of the 4 instances of myeloid leucemia the reduced glutathione quotient (GSH/R.B.C.) was increased, while the absolute amount per 100 c.c. was decreased. In their conclusion they stated: "glutathione . . . showed a marked increase in those cases characterized by pathologic leukocytic stimulation; a marked decrease in those showing bone marrow aplasia, and with no change in those showing physiologic leukocytosis." They said further: "It is suggested that the reduced form of glutathione plays an important role in regulating normal bone marrow activity, and that depletion of this substance in the blood stream or bone marrow may lead to various leukopenic states." Their conclusions and interpretations are open to serious questioning on two scores. First, it does not seem proper to draw conclusions regarding granulopoiesis from the ratio GSH/R.B.C. This ratio, in fact, denies the significance of what glutathione may be present in leucocytes. Second, such conclusions accept the convictions of Hammett, Reimann and others, without any proof and make these convictions a basis for far-reaching deductions.

Respecting the reduced glutathione quotient, Gabbé showed that it was readily increased within two hours simply through producing an anemia by bleeding. Such a variable ratio seems to us to be a shaky foundation for a theory of pathogenesis. Moreover, other workers, as Pickard and Marsden,[15] concluded from their studies that: "there was no quantitative relation between the hemoglobin and the glutathione of the blood. Although the glutathione of the blood is a part of the contents of the erythrocytes its quantity does not follow variations in either the cell count or the cell volume. . . ." Senturia[16] who studied arthritics and found no significant alteration of the blood glutathione decided that: "In my opinion . . . none of these forms of expression (GSH:R.B.C. ratios and the like) contributed added information beyond the figures given for whole blood." Platt[17] examined, among other specimens, the blood from a patient with chronic myeloid leucemia, and the blood from one with chronic lymphoid leucemia. He separated the red and

TABLE IA

(After Platt)

MYELOID LEUCEMIA		LYMPHOID LEUCEMIA
2,900,000	Erythrocytes	1,700,000
576,000	Leucocytes	200,000
82.5	Glutathione whole blood — mg. per cent	19.6
5.0	Glutathione in 100 c.c. plasma — mg.	6.0
72.0	Glutathione in 100 c.c. of erythrocytes — mg.	71.0
151.0	Glutathione in 100 c.c. leucocytes — mg.	67.0

TABLE IB

VARIATIONS OF THE REDUCED GLUTATHIONE OF THE BLOOD WHICH OCCUR WHEN SHED BLOOD IS LET STAND

	CONTROL		AFTER 2 HOURS	
	RED GSH	TOTAL GSH	RED GSH	TOTAL GSH
	MG. PER CENT		MG. PER CENT	
1	31.0	42.0	25.7	40.5
2	33.7	44.7	36.8	46.7
3	30.7	39.9	24.5	38.0
4	40.0	48.5	38.7	49.4
5	37.7	44.8	34.1	43.9

All values are averages of duplicate determinations.

white blood cells, and analyzing them found the results that are reproduced in Table IA. Our own determinations of the GSH content of leucocytes yielded comparable values. Platt also analyzed 100 c.c. of pus, and found 164 mg. of glutathione. These observations, and many others, demonstrating the presence of glutathione in leucocytes, indicate that they should not be ignored in attempting to interpret variations of the blood glutathione. Finally, with respect to interpretation, it seems more desirable to us to attach greater significance to the "total glutathione" content than to the reduced glutathione content of the blood. The two are in some phase of equilibrium, and as we have already indicated, the reduced glutathione may be readily oxidized by atmospheric oxygen with the aid of such catalysts as copper, hemin, and hemochromogens. Thus, if shed blood should be hemolyzed slightly or altered by contaminants in the collecting vessels, the reduced glutathione concentration could change. Table IB illustrates the variation of the reduced glutathione which occurs when the blood sample is allowed to stand at room temperature for two hours before the filtrate is made. The total glutathione varies within the error of the method—about 1.0 to 1.5 mg. per cent. As contrasted with the variability of the reduced glutathione value, the total glutathione is estimated only after a zinc reduction of the filtrate, which eliminates all the oxidized glutathione (GSSG). Actually, the value for glutathione obtained by iodate titration is too high by about 3 to 5 mg. per cent due to the presence of reduced ascorbic acid, thioneine, methionine, etc. The error is fairly constant and is disregarded.

Our own study was undertaken to determine the significance, if any, of variations of the total glutathione content of the blood in blood dyscrasias. The technique used was that of Woodward and Fry.[18] Its accuracy was

TABLE II

DIAGNOSIS	NUMBER PATIENT	NUMBER TESTS	REDUCED GLUTATHIONE MG. %	TOTAL GLUTATHIONE MG. %	NEWCOMER HB. GM. %	R.B.C.	W.B.C.	WINTROBE CELL VOL.
Normal	19	19	33.2 ±1.30	42.16±0.91	15.17±0.221	4.970±0.150	8.01± 0.561	43.2
Myeloid leucemia	8	14	32.38±2.83	40.20±2.58	10.11±0.59	3.712±0.295	75.84±20.85	26.6
Pernicious anemia	12	20	33.92±1.58	44.61±1.50	15.31	4.749	7.23	40.7
Secondary anemia	7	8	27.92	40.48	12.62	4.206	9.16	37.1
Miscellaneous								
Banti's disease, hepatic cirrhosis	2	4	33.3	48.2	15.7	4.86	5.6	41.5
Erythroblastic anemia	1	4	22.9	29.8	9.8	4.73	10.6	34.0
Agranulocytosis	1	1	23.2	38.0	11.7	3.88	1.0	35.0
Polycythemia vera	2	6	65.36	75.16	23.5	8.43	12.2	69.0
	52	76						

checked by adding carefully weighed amounts of glutathione to whole blood. Recovery on five such experiments was 99, 101, 99.5, 100, and 102 per cent, respectively. Seventy-six assays for glutathione were made on the bloods of 52 patients who were seen by us in the medical and hematologic clinics of the University of Chicago. In addition to glutathione estimations, the red blood cell count, white blood cell count, hemoglobin, and cell volume values were all determined on the same sample. For the latter, standard methods were employed. In Table II are listed the types of patients examined and the mean values, with, in some instances, the standard deviations of the results. Strenuous, but unsuccessful, efforts were made to effect some sort of correlation between the glutathione values and the red blood cell count, leucocyte count, hematocrit, and hemoglobin, and between various indices calculated from these determinations. That the glutathione content varied to a certain extent with the erythrocyte and leucocyte counts was obvious from inspection of some of the data where several estimations were made on individual patients. Examples of this visual relationship are presented in Table III.

TABLE III

DATE	RED GSH	TOTAL GSH	CELL VOLUME	HB.	R.B.C.	W.B.C.
Patient 1		*Chronic Myeloid Leucemia*				
1/21	38.0	48.2	36	14.5	4.67	122,700
2/16	35.0	43.5	37	14.5	4.31	15,300
3/16	49.7	56.2	40	16.4	5.15	31,600
9/4	41.5	49.1	32	10.0	4.04	44,700
Patient 2		*Chronic Myeloid Leucemia*				
1/14	55.0	62.5	21	10.1	3.22	310,000
1/20	30.0	43.0	28	8.5	3.19	28,750
2/16	21.7	32.0	25	9.8	3.93	4,200
Patient 3		*Erythroblastic Anemia (Leucemia?)*				
1/19	23.3	27.3	33	10.7	5.40	11,300
2/9	19.7	29.5	41	9.8	4.60	–
2/15	20.5	28.5	35	9.6	4.51	9,200
3/16	28.0	32.8	28	9.5	4.40	11,400
Patient 4		*Pernicious Anemia and Metrorrhagia*				
1/21	33.5	39.0	37	12.6	4.44	6,200
2/11	28.3	39.3	35	12.9	4.21	7,200
3/3	24.5	32.2	30	9.3	3.54	5,900
Patient 5		*Polycythemia Vera*				
9/4	65.0	76.7	65	22.4	9.37	15,200
9/6	–	74.3	67	21.3	8.17	14,300
9/9	54.0	65.0	56	17.8	6.55	16,100

An effort at a tabular presentation of a possible influence of small variations in the leucocyte count was attempted. The result is shown in Table IV. All the values for total glutathione and hemoglobin were listed in seven categories of leucocyte counts regardless of diagnosis. The percentage variation of the average total GSH and the average hemoglobin of each category from the normal control mean value is interesting because of the almost complete lack of consistency.

By inspection of Tables III and IV it is apparent that there is some tendency for the glutathione of the blood to vary in the same direction as changes in the erythrocyte and leucocyte counts. This is not surprising, for

TABLE IV

(A) Categories and Averages

	AVERAGE HB.—GM. %	AVERAGE TOTAL GSH MG. %	LEUCOCYTE RANGE
A	11.70	38.00	1,000- 2,500
B	12.63	42.60	2,600- 5,000
C	15.00	43.17	5,100- 7,500
D	14.46	41.86	7,600- 10,000
E	13.46	37.80	10,100- 15,000
F	9.96	38.92	15,100-100,000
G	10.78	52.44	over 100,000
Normal mean	15.17	42.16	8,010

(B) Percentage Variations of the Averages in (A) From the Normal Mean Values

CATEGORY	HB. PER CENT	TOTAL GSH PER CENT	NUMBER IN CATEGORY	W.B.C. PER CENT
A	-23	-10	1	- 87
B	-17	+ 1	7	- 34
C	- 1	+ 3	18	- 19
D	- 4	- 0.7	11	+ 12
E	-11	-10	9	+ 40
F	-34	- 8	8	+ 510
G	-22	+24	5	+1450

it is generally believed that the majority of the blood glutathione is in the cells. However, there is no evidence to indicate that the quantity in individual cells is constant. There is, further, no positive implication in any biochemical study which would suggest a causation for the variation that occurs.

CONCLUSIONS

1. From a study of blood samples from 52 patients, it appears that variations of the glutathione content in hematologic patients follow crudely changes in the number of formed elements in the blood.

2. When a group of normals are compared with a group of patients suffering from blood dyscrasias, the variations in the glutathione content of the blood are not statistically significant.

3. It is impossible for us to correlate changes in the blood glutathione with any variations of the erythrocyte and leucocyte counts, the hemoglobin, or the cell volume.

4. There is no good evidence which can be deduced from our data to justify any attempt to interpret the pathogenesis of blood dyscrasias on the basis of alterations of the blood glutathione.

5. The current views of the function of glutathione in the body's chemistry are reviewed, and the failure of modern biochemists to attribute to glutathione the role of "a universally occurring chemical stimulus to cell increase in number" is apparent.

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THE "AVERAGE DAILY RETICULOCYTE RESPONSE" DURING THERAPY IN PERNICIOUS ANEMIA*

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THE definite relationship between the level of the red blood cell count in patients with pernicious anemia and the subsequent reticulocyte content of the peripheral blood after therapy was first pointed out by Minot and his associates.[1] Later Riddle[2] developed a formula for the correlation of the initial red blood cell count and the maximum reticulocyte percentage after oral liver extract therapy. Bethell and Goldhamer[3] amplified this, adding data for desiccated stomach and liver extract intravenously. Appreciating the fact that some patients did not reach the calculated peak of reticulocyte production, although the response was otherwise satisfactory, and noting the different lengths of time required for the total "reticulocyte response," Bethell[4] developed a formula to correlate the "area" of the plotted reticulocyte curve with the initial red blood cell count. This was used as an expression of the degree of bone marrow involvement. Bethell's formula added the factor of total time, represented by the length of the base of the figure when the reticulocyte curve was plotted.

*From the Thomas Henry Simpson Memorial Institute for Medical Research, University of Michigan, Ann Arbor.
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TABLE I

The "Average Expected Daily Reticulocyte Percentage" After Treatment in Pernicious Anemia, With Various Initial Red Blood Counts Calculated From the Formula $\frac{20.2 - 4.1\ Eo}{1 + 0.25\ Eo}$

Eo	"R"
0.4	16.9
0.5	16.1
0.6	15.4
0.7	14.7
0.8	14.1
0.9	13.4
1.0	12.9
1.1	12.3
1.2	11.7
1.3	11.2
1.4	10.7
1.5	10.2
1.6	9.7
1.7	9.3
1.8	8.8
1.9	8.4
2.0	8.0
2.1	7.6
2.2	7.2
2.3	6.8
2.4	6.5
2.5	6.1
2.6	5.8
2.7	5.4
2.8	5.1
2.9	4.8
3.0	4.5
3.1	4.2
3.2	3.9
3.3	3.7
3.4	3.4
3.5	3.1

Eo—Initial red blood count in millions per c. mm.
"R"—Average daily reticulocyte percentage.

In an effort to develop a simple relationship between the initial red blood cell count (and therefore by implication, the total number of megaloblasts stored in the bone marrow) and the length of time necessary for blood production to reach an equilibrium (expressed by the number of days after therapy was started, before the reticulocyte delivery reached a relationship comparable to that in health) we have made the following study. The data were taken from an analysis of the complete reticulocyte response of 21 patients with pernicious anemia treated with desiccated defatted stomach tissue, 22 patients treated with liver extract by mouth, and 39 patients treated with liver extract intramuscularly. The patients fulfilled all the requirements for the ideal study of reticulocyte reactions in pernicious anemia and were selected because of absence of complicating factors.

The reticulocyte percentage during the complete reticulocyte response, starting with the day after the treatment was started, and ending with the day (inclusive) on which the per cent fell to 3 or lower, were added. This number was then divided by the number of days represented. This "average daily reticulocyte per cent" is, of course, an arbitrary number, not represent-

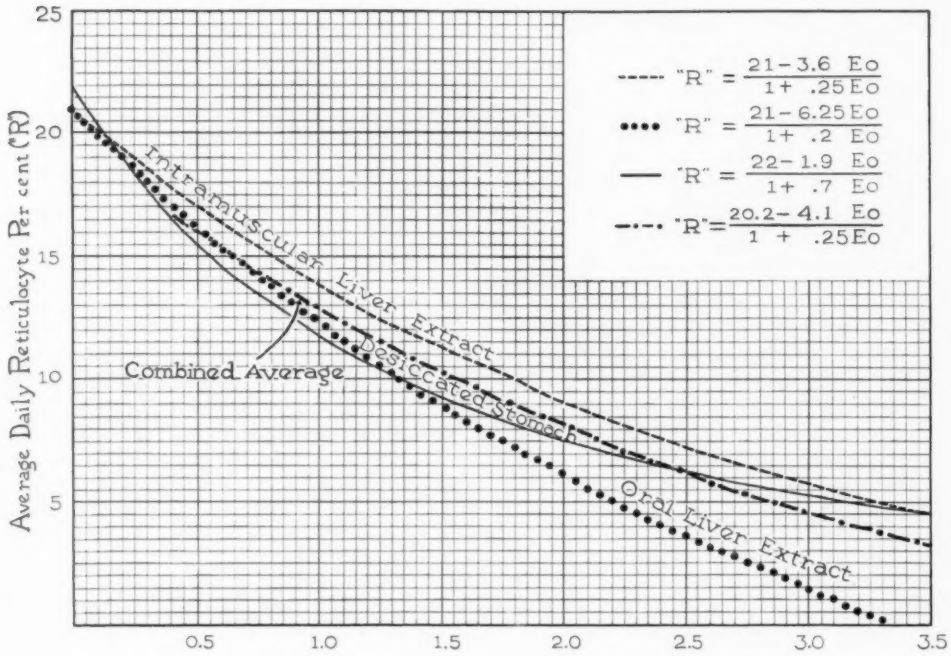

Fig. 1.—Initial red blood cell count in millions per cubic millimeter (Eo).

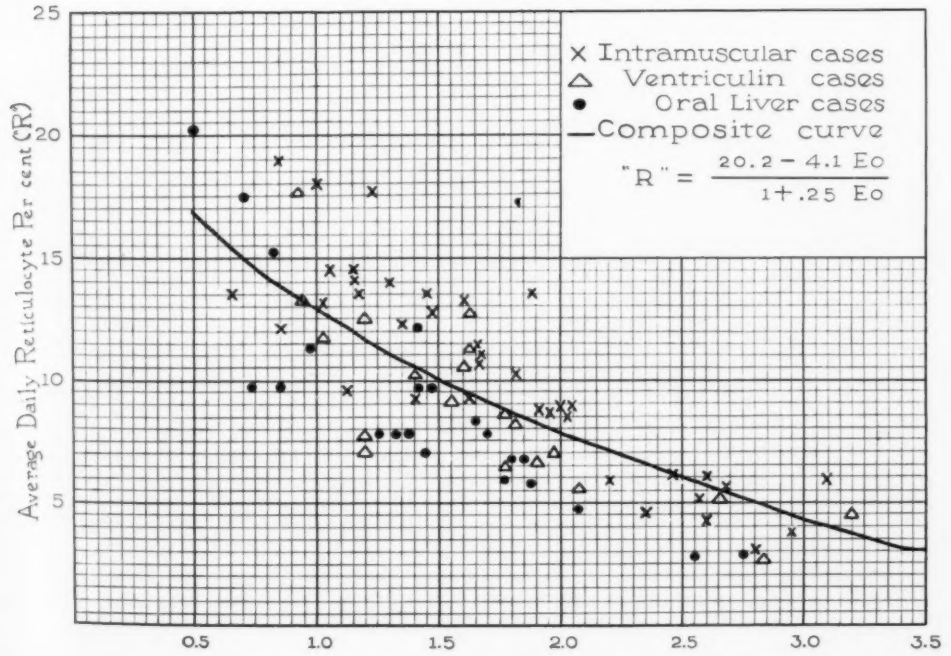

Fig. 2.—Initial red blood cell count in millions per cubic millimeter (Eo).

ing any actual observation, but serving as an index in which a number comparable to the "total" reticulocyte production is correlated with the time element in the process. These numbers were plotted against the initial red blood cell count, and a formula calculated for the curve.

From the data, the total number of days required to complete the "reticulocyte response" were plotted against the initial red blood cell count. There was no correlation evident. Similarly, there was no correlation between the initial red blood cell count and the day on which the maximum reticulocyte per cent was reached.

DISCUSSION

When the observed data of the "average daily reticulocyte per cent" are plotted separately, the trend for the cases treated with liver extract by mouth is slightly lower than that of those receiving desiccated stomach, and this, in turn, is not as high as that of the intramuscularly treated group (Fig. 1). In view of the dispersion of the plotted dots on the curve, it appears to be satisfactory to combine all of the cases in one group (Fig. 2). The formula for the curve which best fits the intramuscular group is:

$$\text{"Average daily reticulocyte per cent"} = \frac{21 - 3.6\ Eo}{1 + 0.25\ Eo}$$

For the desiccated stomach group:

$$\frac{22 - 1.9\ Eo}{1 + 0.7\ Eo},$$

For the oral liver extract group:

$$\frac{21 - 6.25\ Eo}{1 + 0.2\ Eo},$$

For the total group:

$$\frac{20.2 - 4.1\ Eo}{1 + 0.25\ Eo}.$$

SUMMARY AND CONCLUSIONS

1. There appears to be a simple relationship between the level of the red blood cell count on the day that treatment is started in patients with pernicious anemia and the total reticulocyte production, as expressed by the total reticulocyte percentages of each day divided by the number of days required for the total "reticulocyte response."

2. The curve which best fits this relationship has a formula for each point of

$$\text{"Average reticulocyte per cent"} = \frac{20.2 - 4.1\ Eo}{1 + 0.25\ Eo}.$$

3. This arbitrary relationship is an expression of the total reticulocyte production, and, therefore, a measure of the number of cells blocked at the megaloblast stage in the bone marrow of untreated patients with pernicious anemia.

4. There was no simple correlation between the total number of days required to complete the reticulocyte response and the initial red blood cell count.

5. There was no simple correlation between the initial red blood cell count and the day on which the maximum red blood cell count was reached.

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THE PHARMACOLOGIC ANTAGONISM OF METRAZOL AND SODIUM AMYTAL AS SEEN IN HUMAN INDIVIDUALS (SCHIZOPHRENIC PATIENTS)*

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THE antagonistic action of metrazol and the barbiturates has been the subject of considerable work by numerous investigators. The pioneer work of Tartler[12] demonstrated clearly that sodium barbital narcosis was quickly overcome by metrazol and that the convulsant threshold of metrazol was raised about fourfold by sodium barbital. These observations have been confirmed and extended by the work in animals of Mehl,[10] Maloney,[9] Kohn and Jacobi,[7] Biehler,[4] Zipf and co-workers,[13, 14] Axmacher,[2] Albus,[1] Schwab and Jung,[11] Gros and Hofmann,[5] and Jackson.[6] The concept of the "awakening effect" (Koppanyi and associates[8]) has been applied by Barlow[3] to metrazol in explanation of its modus operandi in overcoming the effects of the barbiturates. To my knowledge there is no report in the literature concerning the effects in human individuals of the simultaneous injection of both of these drugs.

The experiments to be described here were primarily designed to test the psychiatric effects of metrazol when combined with sodium amytal. They have relevance to the recently introduced convulsive treatment of schizophrenia and other mental disorders with metrazol. Some of the data obtained seemed to be of sufficient pertinence to the pharmacologic problem of the barbiturate-metrazol antagonism to warrant separate report. The effects of rapid, simultaneous, intravenous injection of combined metrazol and sodium amytal in a group of 5 schizophrenics were studied.†

PROCEDURE

Of the subjects studied, 4, M. C., G. L., W. T., and L. C., were women, and one, L. O., was a male. All were chronically disturbed patients who were part of a large group which I treated for a period of fourteen to twenty-three

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†It may be mentioned that sodium amytal, in dosages sufficient to induce narcosis of greater or less duration, is also used independently in the treatment of schizophrenia.

successive days with metrazol (Bilhuber-Knoll). Metrazol in 10 per cent solution only was used. Two or three days following the cessation of this series, the following steps of the experiment were undertaken in order:

(1) The convulsive threshold of metrazol was redetermined.

(2) The following day and on successive days thereafter, the convulsive threshold dose of metrazol combined with 0.25 gm. sodium amytal (Lilly) was injected. The drugs were mixed together and injected intravenously as rapidly as possible. The amount of metrazol was increased daily until a convulsion occurred.

(3) On the day following the occurrence of the convulsion, 0.25 gm. sodium amytal was injected alone, over a period of two and one-half minutes.

(4) The following days and on successive days thereafter, metrazol combined with 0.5 gm. sodium amytal was injected under the same conditions as (2). The amount of metrazol was increased daily until a convulsion occurred.

(5) On the day following the occurrence of the convulsion, 0.5 gm. sodium amytal was injected alone over a period of two and one-half minutes.

In patient L. O., because of the high convulsant threshold obtained with metrazol combined with 0.25 gm. sodium amytal, the experiments with 0.50 gm. sodium amytal were not carried out.

RESULTS AND CONCLUSIONS

Since in most patients the dosage of metrazol alone must usually be increased after successive injections in order to produce convulsions, it is necessary to evaluate the data in the light of this factor of changing "tolerance." For each of the patients, therefore, a graph is presented of the dosages used and the convulsions produced over the period of from fourteen to twenty-three successive days, during which metrazol alone was administered. On the same graph there are presented the data on the dosages of combined metrazol-sodium amytal after the two-day or three-day interval of nonmedication. The points at which convulsions occurred are also indicated. The effects of the metrazol-sodium amytal mixtures and of sodium amytal alone are written on this portion of the graph in the appropriate places.

From the data in Fig. 1 the following points of significance may be noted:

1. Certain combinations of sodium amytal and metrazol may be injected intravenously at a rapid rate in human individuals with no hypnotic or convulsive effects.

2. Sodium amytal raises the convulsive threshold of metrazol. Conversely, metrazol in convulsive dosages diminishes the hypnotic effect of sodium amytal, even when convulsions are not produced. The upward change in the convulsive threshold of metrazol, when combined with sodium amytal, is far greater than that of metrazol alone.

3. The convulsive threshold of metrazol seems to bear a direct relationship to the amount of sodium amytal with which it is combined. Whether or not the hypnotic effect of sodium amytal bears an inverse relationship to the amount of metrazol with which it is combined cannot be established from these data.

4. In patients in whom relatively small dosages of sodium amytal (0.25 gm. intravenously) alone produce no apparent hypnotic effect, the same amount is effective in raising the convulsive threshold of metrazol.

5. The inference seems clear that the antagonism of sodium amytal and metrazol (as indicated by the neutralization of the hypnotic effects of the

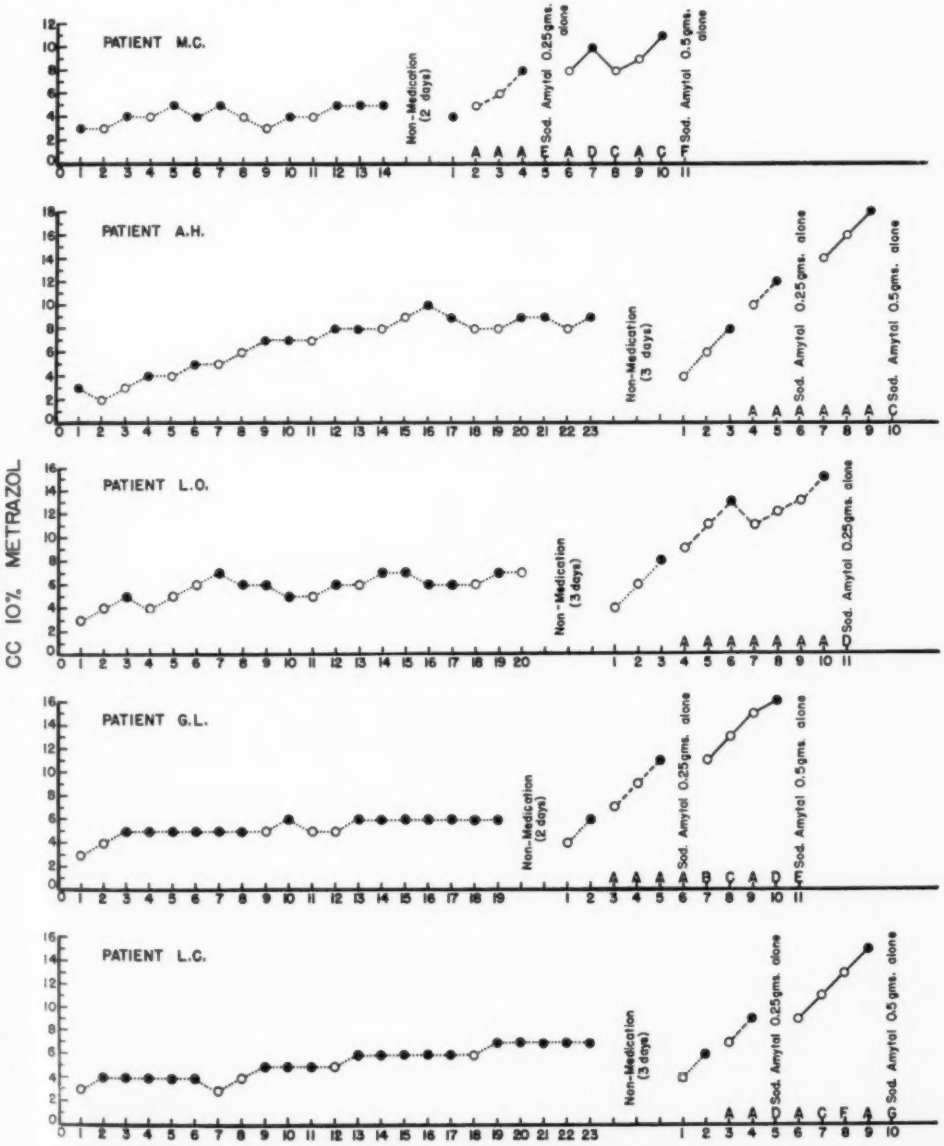

Fig. 1.—Antagonistic effects of metrazol and sodium amytal.

○ Absence of convulsion.
● Occurrence of convulsion.
. . . . Metrazol alone.
- - - - - Metrazol combined with 0.25 gm. sodium amytal.
——— Metrazol combined with 0.5 gm. sodium amytal.

A—No drowsiness.
B—Drowsy rest of day.
C—Drowsy two hours.
D—Drowsy fifteen to twenty minutes.
E—Slept twenty to thirty minutes followed by drowsiness.
F—Slept one to two hours.
G—Slept six hours.

former and the convulsant effects of the latter) is one which may depend upon the action of both drugs on the same centers of the nervous system. There is also the presumption that the speed of action of both drugs must be very nearly the same.

SUMMARY

The mutually antagonistic pharmacologic action of metrazol and sodium amytal has been studied in human individuals (schizophrenic patients) under conditions of rapid, simultaneous, intravenous injection of both drugs. The antagonism consists in the inhibition of the hypnotic effects of sodium amytal by metrazol and in the rise of the convulsant threshold of metrazol by sodium amytal. The efficacy of the antagonistic effect of sodium amytal to metrazol seems to depend upon the relative amounts administered. It is suggested that both drugs probably affect the same centers in the nervous system and at the same rate of speed.

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EXAMINATION OF EFFUSIONS IN TUMOR CASES*

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RECENT experiences and doubts concerning the reliability and practical value of pathologic examinations of effusions in tumor cases prompted the following short survey.

During the first ten-year period of a medium-sized rural hospital, the routine biopsies amounted to about 300; the number of autopsies to about 44 a year. Although the absolute figures have been gradually increasing within the last two years—1937: 512 biopsies, 43 autopsies; first six months of 1938: 209 biopsies, 45 autopsies—the above-quoted averages of the last decade since 1928 still give a fairly accurate measure of the material available for investigation in the pathologic laboratory of the hospital.

An attempt was made in 27 cases either to corroborate or to exclude a tumor diagnosis and eventually to arrive at a conclusion about the cellular characteristics of a given growth through microscopic investigation of chest or abdominal effusions. The examination was carried out in chest fluid in 16 cases, in abdominal fluid in 8 cases; in one patient pleural transudate and ascitic fluid both were available. Once the stomach contents and once the fluid from a punctured cyst of the parotid gland were examined without success. On the whole, the tests have been carried out in 50 instances.

The findings for this survey were ascertained as follows: The available slides were studied, and a tentative diagnosis, based upon an exact histologic description of the cellular peculiarities of the effusion, was made. All the clinical, autopsy, biopsy and operative notes available were collected and compared with the original pathologic description and diagnosis of the respective specimens. A comparison of the two diagnoses proved an almost complete conformity. An attempt to improve originally incorrect or doubtful diagnoses failed.

The procedure recommended originally by Mandlebaum[1] and later by Zemansky[2] was applied with only slight changes in minor details, partly similar to those mentioned and suggested by Foot[3] in 1937. Formalin and Zenker fixation gave identical results. Centrifugation and filtration of the fixed sediment seemed to be slightly superior to repeated centrifugations. It may well be that the less dense packing of the cellular elements in the sediment after only one single centrifugation results in a better dehydration under the treatment with alcohol. Paraffin sections of the sedimented cells were, in our experience, far superior to simple smears of sedimented material, fixed and treated in the fashion of the usual blood stain. Hematoxylin-eosin, eosin-methylene blue, and Wright's stain were of almost equal tinctorial value and sufficient for the purposes of differential diagnosis.

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In reviewing the results of this small series of fairly representative material for judging the diagnostic procedure (see Table I) one recognizes that, as far as differential diagnosis is concerned, negative findings are apparently of less significance than positive results. The latter, marked by the presence of tumor cells in pleural or abdominal effusions, establish the diagnosis beyond any reasonable doubt, whereas in those cases in which the clinical diagnosis leans more towards a nonneoplastic origin of the effusions, the absence of tumor cells in the respective fluids will be only one more link in the whole chain of diagnostic evidence against the presence of a malignant growth. It is easily understood that the diagnostic problem is of especially great significance in those cases in which a pleural or abdominal effusion is caused by a systemic growth of the lymph glands, as, for instance, in Hodgkin's disease, lymphosarcoma, or generalized metastatic involvement of the lymph glands in certain rare cases of carcinoma of the stomach or prostatic gland. The chylous appearance of the fluids in some effusions may eventually add considerably to the uncertainty of the findings.

Two reasons are obvious for the difficulties encountered in the interpretation of the examinations. One rests on the fact that a good number of tumors, although spreading intraperitoneally or intrapleurally, do not give rise to the presence of tumor cells floating within or on the surface of the effusions. The very instructive descriptions and pictures of Quensel[4] discuss this factor in all the necessary detail and show that this possibility always has to be kept in mind whenever a negative histologic finding contradicts otherwise sufficient and striking clinical evidence in favor of a neoplasm.

The other factor is found in difficulties of correctly interpreting the cells encountered in pleural and abdominal effusions. It would lead too far afield to discuss all the possible sources of errors, starting with technical shortcomings; for instance, in consequence of delayed examination of the fluids, necrobiotic cellular changes may have taken place before adequate fixation. It seems to be unnecessary to dwell on these possibilities, since several authors rather recently have described the cellular findings in effusions with much detail, some of them adding excellent and instructive pictures; furthermore they have discussed the above-mentioned possible technical errors (Graham,[5] Quensel,[6] Karp,[7] Merklen and associates[8]). These papers all deal with the importance of correctly differentiating the plaques of more or less swollen or otherwise changed endothelial cells from tumor cells as they are found in practically every effusion of longer duration. But, whereas it seems to be comparatively easy to come to a conclusion concerning the endothelial origin, even of a multinucleated cell, in relying on the multitude of criteria available, it is still more than difficult and very often doubtful to diagnose positively a single tumor cell. Quensel,[6] MacCarthy,[9, 10] and Foot[3] stress the value of the so-called nucleo-nucleolar index, whereas Guttmann and Halpern[11] emphatically deny the diagnostic value of this phenomenon. Although in the present small series it was not possible to carry out correct measurements of the nucleo-nucleolar index, special attention has been paid to the presence of large nucleoli in cells, otherwise suggestive of neoplastic origin. But it was possible in only 2 out of 5 tumor cases with peritoneal or pleural spread, in which tumor cells could be expected and had not been found previ-

TABLE I

CASE NO.	NO. OF EXAM.	DIAGNOSIS	TUMOR SPREAD			EFFUSION		TUMOR CELLS		
			+	?	-	ABDOMINAL	PLEURAL	+	?	-
2	5	Cystadenoma of ovary with metastases	+			+	+	+		
3	6	Endothelioma of pleura	+				+	+		
10	1	Cystadenocarcinoma of ovary	+			+		+		
25	3	Cystadenoma of ovary with metastases	+			+		+		
26	1	Carcinoma of breast with invasion of pleura	+				+	+		
22	2	Adenocarcinoma of pleura	+				+	+		
6	18		6			3	4	6		
1	4	Carcinoma of breast with metastases	+				+		?	
5	1	Small cell carcinoma of ovary	+			+			?	-
8	1	Papillary carcinoma of ovary	+			+			?	
3	6		3			2	1		2	1
16	2	Adenocarcinoma of bronchus		?			+			-
19	1	Carcinoma of lung, hemothorax		?			+			-
20	1	Secondary carcinomatosis of peritoneum		?		+				-
21	1	Undiagnosed malignancy of kidney		?			+		?	-
9	2	Hodgkin's disease or Ewing's sarcoma			-		+			-
13	1	Neoplasma of mediastinum			-		+			-
15	1	Adenocarcinoma of colon			-	+				-
18	1	Primary carcinoma of liver			-	+				-
27	4	Carcinoma of prostate with metastases to lymph glands			-		+			-
11	2	Adenocarcinoma of head of pancreas		?	-	stomach				-
10	16			4	6	3	6		1	9
4	1	Unspecific pleuritis					+			-
6	2	Syphilitic heart disease					+			-
7	1	Chronic pulmonary tuberculosis with pleurisy					+			-
12	1	Arteriosclerotic heart disease				+				-
14	2	Undiagnosed disease of lungs (actinomyces?)					+			-
17	1	Cyst of parotid gland				cyst				-
23	1	Perforated ulcer of stomach, peritonitis				+				-
24	1	Arteriosclerotic heart disease					+			-
8	10					2	5			8
		Summary								
27	50	Tumors: with spread: 9+, 4? Tumors: without spread: 6 Other causes: 8	9	4	6	10	16	6	3	18

ously, to establish (retrospectively) the diagnosis on account of the presence of very large nucleoli. It may be that a correct micrometry of a wealth of material perhaps gives with increasing experience a greater feeling of security in the interpretation and evaluation of this special criterion. So far, however, it seems somewhat audacious to arrive at a positive tumor diagnosis in an otherwise doubtful specimen only on account of one definite cellular characteristic, namely, the very large nucleolus. Since multinucleation, mitoses, giant cavities, signet ring formation, and tinctorial anomalies may be observed as well within cells of endothelial origin, the most valuable criterion is still the presence of typical tumor elements; not in the form of single cells, but of cell clusters, acini or other larger histologic entities, as used in the common pathologic routine diagnosis of tissues.

It may be added as completion of Table I, that the diagnosis could be verified both by autopsy and by biopsy and operation nine times. Only in those 4 tumor cases where the metastatic spread to pleura or peritoneum is noted as questionable (Nos. 16, 19, 20, and 21 of Table I), the final proof for the correctness of the diagnosis was missing. The same holds true for 5 out of the 8 non-neoplastic cases.

A glance at Table I shows that in applying the above-mentioned criteria the diagnosis could be established at once in 6 out of 9 patients in whom the kind of pleural or peritoneal spread made the presence of floating tumor particles or cells more than probable. In 2 of the remaining 3 cases, the interpretation of the cellular findings was doubtful, but here, as already pointed out, the presence of large nucleoli, and probably even better the determination of the nucleo-nucleolar index, should have pointed in the direction of the correct tumor diagnosis. To these 3 negative, or more exactly questionable, results must be added 5 more cases, in which a pleural or peritoneal infiltration was very likely, although no autopsy findings were available to corroborate the diagnosis. Within the effusions of these cases no tumor cells could be discovered either. However, in one the verification of cells suggestive of neoplastic origin was at least suspected and jotted down as questionable, but not sufficient for a definite diagnosis (Case 21). On the other hand, in no case was a tumor diagnosis made where later at autopsy or operation it was conclusively shown that the presence of malignant cells within the fluid would have been highly improbable, if not impossible, on account of the absence of a peritoneal or pleural neoplastic involvement. In taking into account these cases and in adding others, where a nonspecific pleurisy or an ascites in a case of cardiac cirrhosis gave rise to the effusion, which likewise showed negative results, one sees that the diagnostic value of the method is not as small as first assumed. Although, naturally, negative finding are, as pointed out before, of far less diagnostic significance for the clinician, the danger of wrongly interpreting the abnormal cells of endothelial provenience as neoplastic cells is apparently negligible.

Two cases out of the foregoing series may be described briefly because of post-mortem findings which were of special interest.

No. 27, J. St., was a male patient, 48 years of age, who came to the hospital after a half year's illness. He had first noticed a swelling of his left leg which, in the course of the next few months, became gradually cyanotic and dyspneic. On admission a huge mediastinal mass and also a generalized swelling of his inguinal, cervical, and axillary lymph glands was discovered as cause of his symptoms. At the time of his sudden, rather unexpected death, the clinical diagnosis was lymphosarcoma or Hodgkin's disease. The possibility of a primary carcinoma of unknown origin with metastatic spread to the lymph glands was also discussed. At no time were any malignant cells found in the pleuritic effusion, although the endothelial cells, which were present in great numbers, single and in plaques, were first difficult to differentiate. At autopsy the presence of large masses of firm, yellowish gray lymph glands at the above-mentioned locations seemed to be in favor of a primary malignant disease of the lymph glands. The fact that the neoplastic masses in the pelvis invaded the seminal vesicles was noticed, but did not change the preliminary, gross diagnosis. Microscopic examination, however, revealed that not a primary systemic process of the lymph glands caused the growth, but a metastatic invasion originating in the prostatic gland. This kind of tumor spread, limited to the lymph glands, is known but very rare.

No. 5, V. H., was a 47-year-old married woman, who died eight and one-half months after the onset of vaginal bleeding caused by a malignant tumor of the ovary. In spite of profuse spread through the peritoneal and pleural cavities, it was not possible to trace tumor cells within the abdominal effusion. Histologically, the tumor turned out to be a so-called small cell carcinoma of the ovary. Among many metastases found at necropsy and microscopic study, one was encountered within the mitral valve. A careful search of the literature did not disclose the description of a similar location of a tumor metastasis.

SUMMARY

1. Pathologic examination of pleural and abdominal effusions in tumor cases has been tried out routinely in the material of a medium-sized rural hospital.

2. Attention is called to the fact that even in the presence of an intraperitoneal or intrapleural tumor spread, malignant cells within an effusion cannot be expected in every case, their occurrence depending upon the special conditions of the neoplastic growth.

3. The difficulties arising from the interpretation of the cellular findings and the necessary differential diagnosis between cells of endothelial and neoplastic origin are briefly discussed.

4. The number of cases in which tumor cells could be demonstrated was comparatively small in this series. However, the absence of any misinterpretation of endothelial cells as tumor cells, gives a somewhat increased diagnostic value to the negative findings.

5. One case of prostatic carcinoma with metastatic spread exclusively to the lymphoglandular system, and one of an ovarian carcinoma with a metastasis within the mitral valve, are briefly described.

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THE CONCENTRATION OF ACACIA IN THE SERUM, ITS RATE OF EXCRETION, AND ITS EFFECT ON THE COLLOID OSMOTIC PRESSURE FOLLOWING INTRAVENOUS INJECTION IN CASES OF CIRRHOSIS OF THE LIVER*

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THE widespread use of acacia as a substitute for blood has focused considerable attention on this substance and its probable physical and chemical actions in the organism. The purpose of this study was to determine the concentration of acacia in the blood, its rate of excretion, and its effect on the colloid osmotic pressure following intravenous injection into man.

It has been established recently that acacia cannot be readily metabolized by the animal body, but that it is deposited in the liver, spleen, and other organs following intravenous administration (Andersch and Gibson,[1] 1934). Others have presumed that deposition of this substance in the liver accounts for the decrease in the levels of plasma protein sometimes observed following the intravenous administration of this agent (Dick, Warweg, and Andersch,[2] 1935).

Acacia disappears rather rapidly from the blood stream, but Keith, Power, and Wakefield[3] (1935) have been able to detect traces of acacia in the blood serum of man three years after its injection. There has been a lack of adequate data on the rate of disappearance of acacia following intravenous injection, and the data of Gasser, Erlanger, and Meek[4] (1919) on rabbits, of Huffman[5] (1929) on man, and of Peoples and Phatak[6] (1935) on dogs, are about all that are available.

METHODS

The colloid osmotic pressure, the values for the serum proteins (albumin and globulin), and the rate of excretion of the injected acacia were determined in three cases of cirrhosis of the liver.

The concentration of acacia in serum and urine was determined by a precipitation method which has been described in preliminary form by Power[7] (1937). The details of the method will be published elsewhere. For serum the procedure involves, first, removal of proteins by means of trichloracetic acid, and, second, isolation of acacia from the protein-free filtrate by means of acetone. The acidity of the serum filtrate should be equivalent to about 8.5 per cent trichloracetic acid. Five cubic centimeters of acetone is mixed with 2 c.c. of filtrate in a moderately pointed centrifuge tube, and the mixture is allowed to stand an hour or more in the refrigerator. The precipitate of acacia is separated in the centrifuge, washed with acetone, and dried. It is then

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oxidized with a known amount of potassium dichromate in the presence of concentrated sulfuric acid, after which the excess dichromate is determined iodometrically. It has been found that purified acacia that has been isolated and treated in this manner requires, on the average, 2.72 c.c. of 0.05 normal solution of dichromate per milligram for complete oxidation. The results of analyses may be calculated with the aid of this factor.

Acacia, as determined by this method, obviously represents a substance which has not become so modified in the body as to lose its property of precipitating in the presence of acetone.* If the acacia hydrolyzes to some extent, the simpler molecules formed might be partly metabolized or partly excreted in the urine. Qualitative experiments have indicated, in fact, that subsequent to intravenous injection of a solution of acacia, the urine may contain more furfural-yielding substances than can be accounted for by the amount of "acetone precipitable" acacia present. It may become of considerable interest to compare the excretion of "precipitable" acacia with that of total furfural-yielding substances in future studies of this sort.

The acacia employed in each case was a 6 per cent (with 0.9 normal sodium chloride) solution that had been sterilized in the autoclave. Mention is made that these solutions were autoclaved because this process supposedly increased the colloid osmotic pressure of the solution (Dodds and Haines,[8] 1934). The colloid osmotic pressure of the blood serum was measured by a modification of the membrane bag arrangement of Starling[9] (1896), Adair[10] (1928), Keys and Taylor[11] (1935), and others. The details of the method will be presented elsewhere, but a few essentials may be given here. The serum is diluted with an equal volume of a phosphate buffer solution at pH 7.4, which is approximately isoionic and isoosmotic with the serum. Small, rather rigid collodion sacs, which have been previously tested and proved to be protein tight, are used. The same buffer solution is used for the external medium and the entire system is kept at 0° C. Usually, equilibration is complete within forty-eight hours, but in all observations in the present series the osmometers were observed for some days longer. At the end of equilibration the amount of nitrogen in both the inner and outer fluids was determined, and the result compared with the values for the protein and nonprotein nitrogen of the original serum. The values for colloid osmotic pressure were corrected to the original concentration of the proteins in the serum by means of standard protein dilution curves. All determinations were made in duplicate. The mean colloid osmotic pressure of the serum obtained from thirty normal persons was found to be 371.3 mm. of water, with a probable error of ±5.38. The variability, as measured by the standard deviation, was 43.7 mm. of water.

RESULTS

Following intravenous injection of a 6 per cent solution of acacia, the colloid osmotic pressure of the blood serum is assumed usually to be elevated only for forty-eight hours (Amberson,[12] 1937). Fig. 1 adequately supports this statement. It is interesting that the value for the total protein of the

*It might be well to mention that the presence of acacia interferes in determinations of serum sulfate performed by benzidine methods in which acetone or alcohol is used as the precipitation medium (Keith, Power, and Wakefield, 1935).

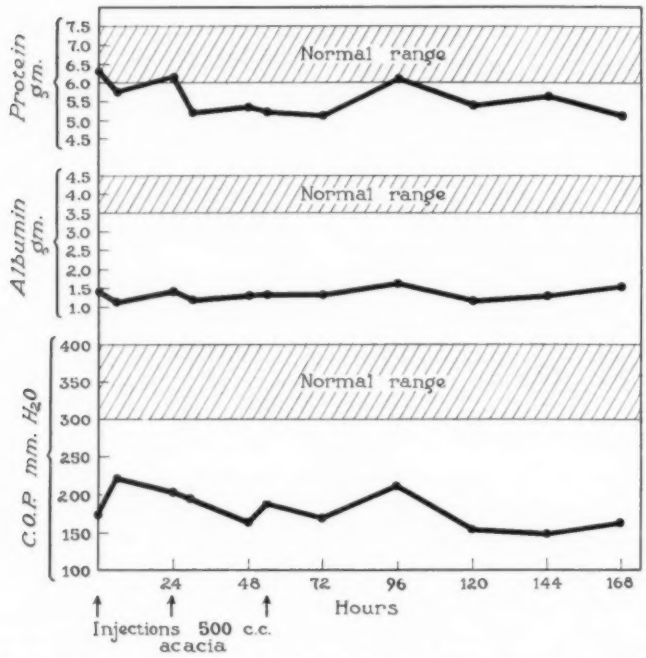

Fig. 1.—Effect of multiple injections of acacia on the colloid osmotic pressure and on the concentration of serum protein of man.

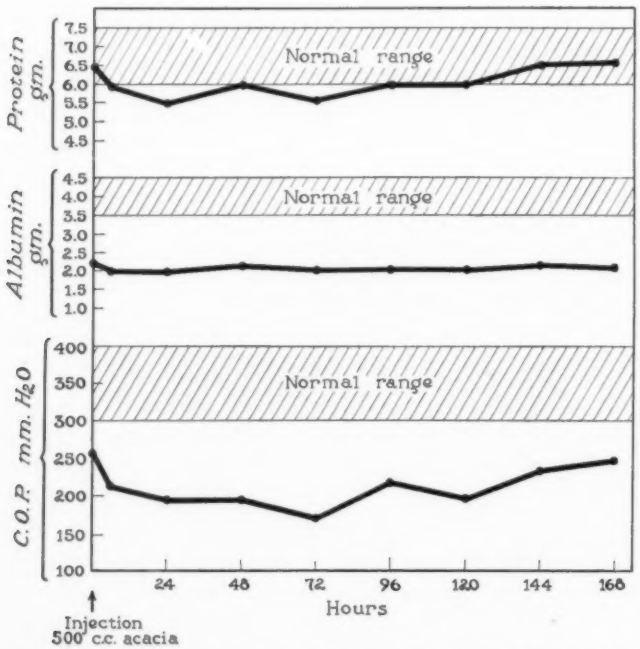

Fig. 2.—Effect of single injection of acacia on the colloid osmotic pressure and on the concentration of serum protein of man.

blood serum was diminished following injections of acacia, but that the albumin fraction was affected little. Similar results have been recorded by Heckel[13] (1938) and his associates, following large injections of acacia into dogs.

In the case summarized in Fig. 2, there was only one injection of 500 c.c. of acacia. The colloid osmotic pressure of the blood serum in this instance was not maintained above, or at, the basal level of the patient for any appreciable period of time.

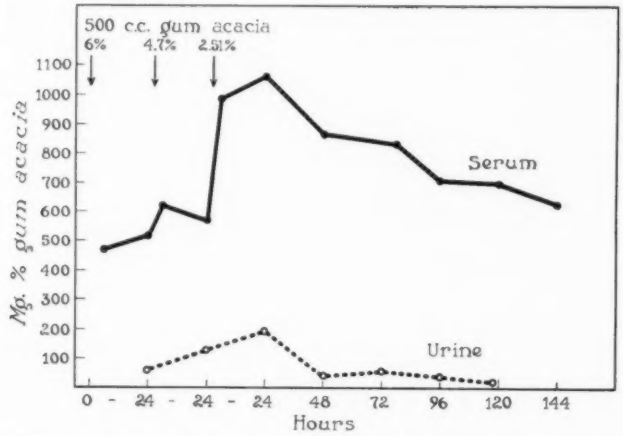

Fig. 3.—Concentration of acacia in serum and urine following multiple injections of acacia.

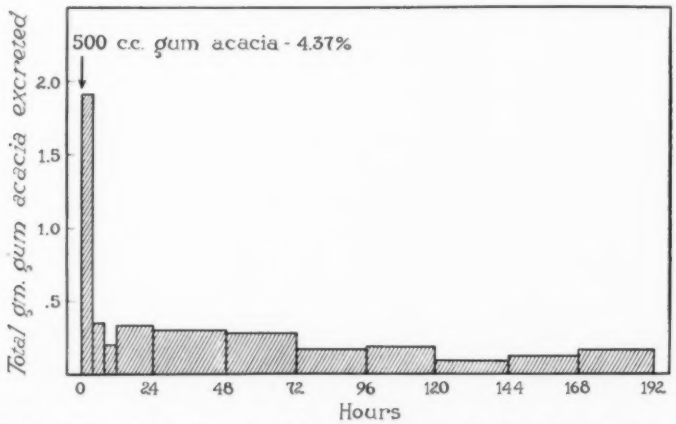

Fig. 4.—Urinary excretion of acacia following single injection of acacia.

It is frequently stated in the literature that the serum proteins, particularly the albumin fraction, fall to a low concentration in man and animals following the intravenous injection of solution of acacia (Dick and others, 1935; Heckel, 1938). Only in the third case, similar otherwise to the case represented in Fig. 1, did the concentration of albumin decrease appreciably following this procedure, but at no time was this reduction of the magnitude reported in the literature.

Fig. 3 represents graphically the concentration of acacia in the blood and urine of a patient with cirrhosis of the liver, following the intravenous injection of acacia. There appears to be some cumulative effect following the third

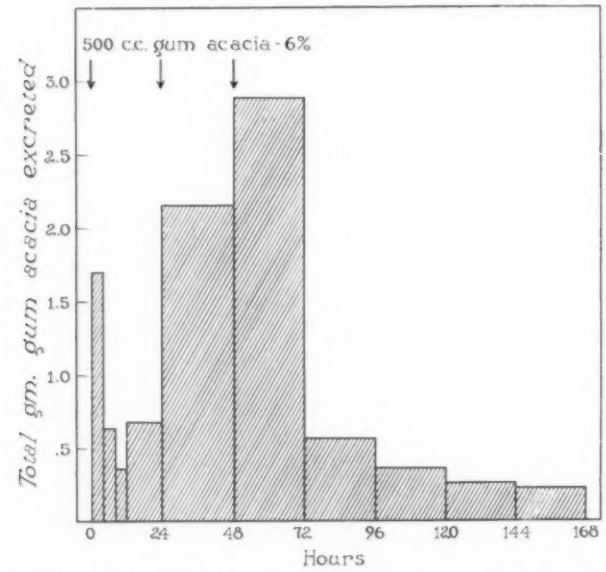

Fig. 5.—Urinary excretion of acacia following multiple injection of acacia.

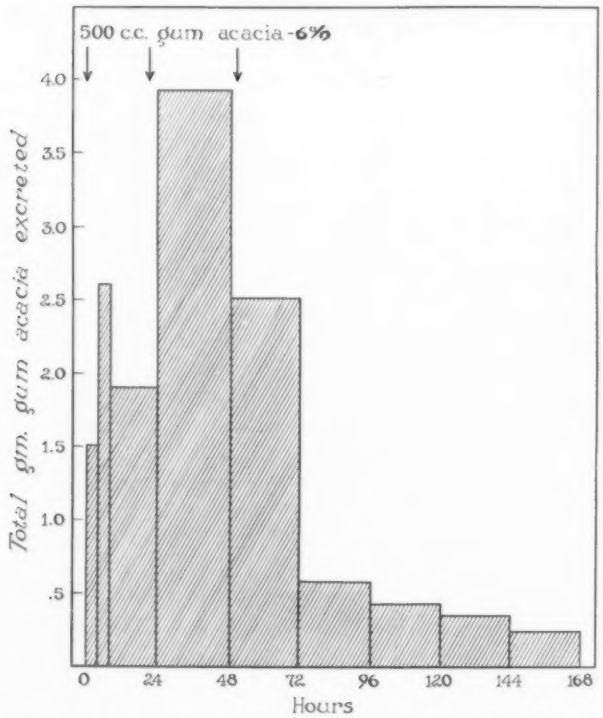

Fig. 6.—Urinary excretion of acacia following multiple injection of acacia.

injection of acacia. The colloid osmotic pressure of the serum does not remain elevated in proportion to the concentration of acacia in the blood, probably because the smaller particles of acacia are excreted rapidly in the urine or diffuse rapidly into the tissues or some other depot.

In Figs. 4, 5, and 6 the excretion of acacia in urine following the injection of various quantities of acacia is shown graphically. It will be seen that, for the period of time included in the graphs, the quantity of acacia excreted by the two patients who received multiple injections was, in each case, less than 20 per cent of the total amount of acacia injected, and that most of the acacia excreted appeared in the first three days following its administration. The patient who received only one injection excreted somewhat more than 20 per cent of the quantity injected during the period of observation. The acacia excreted in the urine probably is composed mainly of the smaller particles which have passed through the membrane of the glomerulus. This loss of osmotically active particles in the urine, together with reduction of the quantity of acacia in the serum as a consequence of other means of disposal in the body, such as storage, probably accounts for the inability of injections of acacia to maintain an elevated colloid osmotic pressure for any long period of time.

SUMMARY

Administration of acacia to man under the conditions of these observations results in a short, temporary rise in the colloid osmotic pressure of the serum. The concentration of acacia in the serum remains high for a much longer period of time than does the colloid osmotic pressure. This discrepancy probably is the result of the rapid loss of osmotically active particles of acacia through the urine, or into the tissues or some other depot.

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RAPID CONVERSION OF TRYPTOPHAN TO INDOL BY RESTING BACTERIUM COLI*

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IN PREVIOUS communications [1, 2] have shown that fresh, uncontaminated urine of the majority of human subjects contains an indol precursor which in all probability is free tryptophan, but no indol as such. This work, which has been confirmed by Carnes and Lewis,[3] suggests an approach to the study of free tryptophan in urine and other substances. Such study in the past has been hampered by the lack of sufficiently sensitive tests as well as by the difficulty in distinguishing free tryptophan from that in a combined state. By utilizing the principle employed in the experiments mentioned above, it should be possible to avoid these difficulties.

The principle combines selective conversion of free tryptophan into indol and subsequent analysis of the end product. The conversion is brought about biologically by the activity of bacteria. As previously carried out, this involved the growth of *Bacterium coli* in the test substances. Certain obvious disadvantages are inherent in such a method, and the present work was done in the attempt to avoid some of them by adapting to this purpose a "resting" bacterial suspension which resembles a chemical reagent rather closely.

Woods,[4, 5] using a suspension of washed *Bacterium coli*, was able to recover from tryptophan solution nearly 100 per cent of the theoretical yield of indol. The washed growth from 20 Roux bottle cultures was suspended in 20 c.c. of Ringer's solution and added to 100 c.c. of 0.02 per cent tryptophan solution in phosphate buffer of pH 7.2. Incubation was at 37° C., with continuous aeration. Indol was recovered very shortly after the incubation was begun, but complete conversion required from sixteen to thirty-six hours. He studied the action of suspensions on various indol derivatives also, and found that only one, β-indol pyruvic acid, was capable of being converted into indol, and that in small amounts under special circumstances.

Happold and Hoyle,[6] using a technique somewhat similar to that of Woods, obtained essentially the same results. These authors were able also to prepare killed suspensions of the bacteria that were almost equal in conversion potency to the unkilled. In their studies conversion required up to forty-eight hours.

The results which I obtained substantiate the essential findings of the above-mentioned works, and show further how a modified technique not only increases the speed of the reaction to a point where it is almost instantaneous, but also simplifies the procedure in such a way that it is more readily available for certain purposes. The results show also a possible fallacy in the assumption of Woods that *Bacterium coli* does not decompose indol.

*From the Buffalo General Hospital and the University of Buffalo Medical School.
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METHODS

In general, the technique employed was that used by Quastel[7] and, more particularly, that used by Vaughan and Hubbard[8] in studies of the destruction of reducing sugars.

The stock strain of *Bacterium coli* known to produce indol actively was grown for twenty-four hours on beef juice agar slants in tubes 1 inch in diameter. The growth was removed with a small volume of physiologic saline and washed repeatedly in saline by centrifuging until the washings gave a negative test for indol. The second washing always gave a negative test, but the organisms were washed three times as an added precaution. Before the final washing, the suspension was divided among a series of tubes in such a way that an appropriate volume of packed sediment would be obtained (usually 0.2 c.c.). After centrifuging, the supernatant liquid was drained off and the tubes containing the bacterial sediment were ready to receive the test fluid. This was added in 3 c.c. quantities and the sediment evenly suspended in it. After a suitable incubation period, the tubes were centrifuged again and the supernatant liquids transferred to small agglutination tubes to be tested for indol as follows: To the 3 c.c. of test liquid were added 1.5 c.c. of petroleum ether. The tube was inverted fifty times to insure uniform extraction of indol. One cubic centimeter of the clear ether layer was then transferred to another tube, and 0.5 c.c. of Ehrlich's reagent was added. The contents were agitated by tapping the tube with the finger until the maximum color was produced in the ether layer.

EXPERIMENTS

I. The first experiment was an attempt to convert tryptophan into indol rapidly when the former substance was dissolved in substrates favorable for bacterial growth. For this purpose the following solutions were employed: (a) tryptophan broth, (b) peptone water, (c) meat juice broth containing the usual amount of peptone, (d) urine, and (e) ascites fluid. A liberal amount of bacterial sediment was added (0.5 c.c.), and the tubes were incubated for twenty minutes at 37° C. In each instance a positive test was obtained.

II. In the second experiment solutions of tryptophan that failed to support a vigorous growth of the organisms were examined. Varying dilutions of tryptophan in distilled water were tested in duplicate. One set was seeded with the organism as in the usual cultural technique, and incubated for forty-eight hours at 37° C. Tests for indol were uniformly negative. The other set was tested in the manner described in the first experiment. Indol was detected in dilutions from 1:1,000 to 1:2,000,000. When the tryptophan was dissolved in physiologic saline and tested in the same way, indol could be detected irregularly only by the cultural technique, while it could be demonstrated in high dilutions by the "resting" bacteria method.

III. The third experiment was a rough attempt to analyze a few of the many factors entering into the reaction.

A. Time: Dilutions of tryptophan from 1:1,000 to 1:3,000,000 in distilled water were treated with the bacterial sediment in duplicate. Incubation was

at room temperature. The first set was incubated for five minutes. The highest dilution giving a color reaction was 1:200,000. The second set was incubated for twenty minutes. The positive test was obtained in a dilution of 1:1,000,000.

B. Temperature: The time of incubation was constant (five minutes). Three temperatures, 0° C., 20° C., and 37° C., were used. All operations, including centrifugation, were carried out as nearly as possible at the given temperature. The results were as follows:

0° C. Negative tests in all dilutions
20° C. Positive test in dilution of 1:200,000
37° C. Positive test in dilution of 1:1,000,000

C. Volume of Bacterial Sediment: A tryptophan dilution of 1:100,000 in physiologic saline was incubated for five minutes at 37° C. in tubes containing varying amounts of bacterial sediment and examined for intensity of color produced in the indol test. The maximum color was produced with any amount of sediment from 0.2 c.c. upwards. Using 0.1 c.c., the color was very faint. Prolonged incubation with such small volume increased the color intensity somewhat.

D. Effect of Glucose: As shown by Logie,[9] the amount of free indol found in a peptone water culture of *Bacterium coli* is diminished if 1 per cent of glucose is added before inoculation. The same result was obtained in the present experiment with "resting" organisms. To one of two tubes of 1:100,000 tryptophan in physiologic saline was added 1 per cent of glucose. Both solutions were treated with bacterial sediment for five minutes at 37° C. The maximum color developed in the indol test upon the solution to which no glucose was added. With the glucose solution a very faint, though distinct, color resulted.

IV. The fourth experiment was an attempt to quantitate the amount of indol produced.

A. Colorimetric comparison between the intensity of the reaction in the test solution and that of solutions of pure indol: On the basis of the work of Logie[9] that indol is utilized to some extent by the bacteria, it seemed probable that this method would prove unsatisfactory. However, it was not known that the amount utilized under the conditions of our tests would be sufficient to exclude this method. In order to study this point the following experiment was conducted:

A series of dilutions from 1:1,000 to 1:4,000,000 of pure indol in physiologic saline was prepared in duplicate. The first set was treated with bacterial sediment. Both sets were incubated for twenty minutes at 37° C. and then tested in the usual manner for indol. In the treated set the highest dilution giving a positive test was 1:500,000, and in the untreated set it was 1:3,000,000. This result indicated sufficient removal of indol as to preclude this direct method of quantitation.

B. Colorimetric comparison between the intensity of reaction in the test solution and that obtained in solutions of pure tryptophan of known strength treated in an identical manner at the same time.

1. Comparison of different sets of solutions of known strength: When a series of dilutions of pure tryptophan was prepared in duplicate and both sets treated in the same way at the same time with bacterial sediments of the same batch, not only was the highest dilution giving a positive test identical in the two sets, but the corresponding tubes of each set showed reactions of the same intensity. However, tests carried out on different days or with different batches of bacterial sediment varied considerably. It was obvious, therefore, that no permanent color standard could be devised and that the standards must be prepared at the same time with identical bacterial suspensions and technique.

2. Quantitation of the indol precursor in body fluids and excretions on the basis of tryptophan equivalents: In the manner already described, a series of dilutions from 1:1,000 to 1:4,000,000 of pure tryptophan in physiologic saline were prepared side by side with samples of fresh urine and body fluid. All were treated for twenty minutes at 37° C. with the same amounts of bacterial sediment from the same batch. Each tube was then tested for indol, and the intensity of color produced in the test samples was compared with that present in the graded series of tryptophan solution.

The results, which are described elsewhere in part,[2] showed that the tests in different specimens varied from negative to an intensity greater than that given by the 1:1,000 solution of tryptophan (0.1 per cent).

SUMMARY AND DISCUSSION

The results of these experiments have shown that the "resting" bacteria technique is readily adapted to the study of low concentrations of free tryptophan. Under optimal experimental conditions the conversion of tryptophan into indol was extremely rapid, being detectable almost instantaneously and maximal within twenty minutes. The method was applicable not only to substrates that normally support a vigorous growth of bacteria, but also to many substrates which, for lack of nutritional or electrolytic requirements, fail to support such growth.

In general, the results support the findings of Woods[4, 5] and those of Happold and Hoyle,[6] referred to earlier in this report. However, certain notable differences are evident. It will be seen, for example, that the speed of the reaction was much greater in the present experiments than in those of the other workers. In attempting to explain this marked difference two possibilities present themselves. The first concerns the indol-producing activities of the various cultures employed. While it cannot be said definitely that my strain was more active than those of the other investigators, such a possibility cannot be excluded, since it is known not only that the strain used in the present experiment was extremely active as compared with other strains, but also that this same strain showed considerable variability in its activity from one culture to another.

A second possibility concerns the proportions of bacterial substance to substrate. In the present work optimal results were obtained when the proportion of packed bacterial sediment to total volume of suspension was at least 1 to 16. On this basis it would be necessary to use 7.5 c.c. of packed sediment in the type experiment of Woods. Whether such a large amount was obtained from 20 Roux bottle cultures cannot be ascertained from the text of his paper.

It should be noted also that the results of one of the present experiments apparently disagrees with Woods' assumption, based on the work of Herzfeld and Klinger,[10] that *Bacterium coli* does not decompose indol, and supports the opposite finding of Logie.[9] While it is impossible from my results to state that indol is actually decomposed, it is certain that appreciable amounts are removed from solution in the heavy suspensions used. It might be asked whether part of the discrepancy between the actual and the theoretical yield of indol in Woods' experiments might not be due to its removal by the organisms rather than entirely to loss from aeration and distillation as suggested.

As pointed out before, this loss of indol was in part responsible for the adoption of a quantitative procedure in terms of tryptophan equivalents rather than the direct quantitation of produced indol in the present work.

CONCLUSION

The so-called "resting" bacterial suspension technique is adaptable to the identification and rough quantitation of free tryptophan found in urine and body fluids.

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LIPID CHANGES IN NERVE DEGENERATION IN AVITAMINOSIS A*

Lipids of Sciatic Nerve in Avitaminosis A

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NERVE DEGENERATION IN AVITAMINOSIS A

SINCE Hart and his co-workers[1] noted muscular incoordination in swine on a diet that has since been shown to be deficient in vitamin A, several workers have investigated quite thoroughly the histologic changes that occur in the nerves in this condition. Mellanby, by the use of staining methods, demonstrated that the changes take place in the myelin sheath, and concluded that they differ only in degree from typical Wallerian degeneration.[2, 3] This work was confirmed by Zimmerman.[4]

In 1934 Sutton and his co-workers[5] published a comprehensive study of nerve degeneration in white rats on vitamin A deficient diets. These authors criticized the staining methods of histologic examination and examined frozen sections in the polarizing microscope. They quoted Cramer and Lee[6] to show that cholesterol and the phospholipids are anisotropic to polarized light and that the triglycerides and fatty acids are isotropic. By this method, they demonstrated the disappearance of anisotropic material in the myelin sheath and verified Mellanby's conclusion that vitamin A degeneration presents a similar picture to Wallerian degeneration in the peripheral nerves. They were able to detect these changes much sooner than by ordinary histologic procedure.

Recently, Helen Gillum and Ruth Okey[7] were able to find no significant variation in the lipid content of brains or any other tissue of white rats on a vitamin A deficient diet.

The nerves used in the present study were obtained from rats used by Dr. T. S. Sutton, who, with Dr. H. E. Setterfield, has been continuing his work on nerve degeneration. Dr. Sutton has published a complete account of the manner in which these animals were depleted of vitamin A, the grade of standard rat used, and the diet of the controls.[5]

There are, in the literature, three methods for the partition of the lipids in a small amount of nerve material.[8-10]† However, none of these make provision for the determination of triglyceride esters or of bound cholesterol. Furthermore, the last two methods are extremely complicated, and all are open to the criticism that the material is dried before extraction.

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†Since the completion of this study, a paper by E. Kirk has appeared in J. Biol. Chem. 123: 623, 1938, describing a method of lipid partition which might readily be adapted to nerve material.

In one series of analyses in the present paper, cholesterol was partitioned into free and esterified, and conjugated lipids were separated into lecithin, cephalin, sphingomyelin, and cerebrosides. A large drop was noted in the saturated lipids, and a smaller one in the unsaturated lipids. Cholesterol remained unchanged. None of the changes, however, were significant according to the rule that, to be significant, "a difference in means must be at least one and a half times the sum of the mean deviation from the respective means."[11] Since Dr. Sutton's study with the polarizing microscope has shown that the nerves do not all degenerate to the same degree, the large mean deviation is understandable. Nevertheless, the sum of the fatty acids of sphingomyelin and the cerebrosides showed a difference that would be significant. Also, since it was evident that both fractions of the saturated and unsaturated lipids changed at about the same rate, another series of analyses was made in which the conjugated lipids were partitioned into saturated and unsaturated fractions only. There was a significant drop in the percentage of saturated lipids.

METHODS

The sciatic nerves were carefully excised so as to remove all adventitious fat and connective tissue. Upon excision, the material was placed in a small vial previously saturated with water vapor by means of a piece of moistened filter paper. At no time were the nerves kept thus for more than an hour. They were weighed as soon after excision as possible, the weight of the two nerves being usually between 50 and 100 mg.

After weighing, the nerves were transferred to a small mortar and finely ground with five or six times their weight of clean silica sand. The powder was easily transferred to a 50 ml. Erlenmeyer flask with the aid of a small spatula. A little powder remaining was washed into the flask with three 3 ml. portions of 95 per cent alcohol and finally with a little ether.

Extraction.—The alcohol-ether mixture was boiled on the steam bath for about a half hour. A 2 inch funnel was put in the flask as an aid in refluxing and as a protection against possible bumping. The solution, after settling of the powder, was carefully decanted into another 50 ml. Erlenmeyer flask. The extraction was repeated twice with 95 per cent alcohol and twice with absolute alcohol. Finally it was extracted twice with chloroform. About 5 ml. of solvent were used each time and boiled for not less than fifteen minutes, or while the previous extract was evaporating to a small volume.

The lipid extract contained, likewise, some pulverized sand and nonlipid material. It was extracted four times with 5 ml. portions of boiling chloroform. The extracts were filtered, in their turn, into a 15 ml. graduated centrifuge tube and boiled to about 0.5 ml. A short piece of melting point tube fused to a thin glass rod was used as an antibumper.

Precipitation of the Conjugated Lipids.—Three drops of a saturated solution of strontium chloride in alcohol were added to the chloroform solution of the total lipid, and then 7 ml. of acetone. The mixture was allowed to stand about one hour, after which time the tube was centrifuged. The supernatant solution was transferred to a 50 ml. Erlenmeyer flask, and the conjugated lipids washed twice with 3 ml. volumes of acetone.

Determination of Free Cholesterol.—The acetone solution and washings from the precipitated phospholipid were evaporated to dryness and the lipid dissolved in 5 ml. of petroleum ether. Ten milliliters of a 0.2 per cent solution of digitonin in 50 per cent alcohol were added, and the mixture evaporated to dryness on the steam bath, out of contact with active steam.

After the flask had cooled, bound cholesterol and neutral fat were extracted with several small portions of cold petroleum ether, the extracts being transferred to a 50 ml. Erlenmeyer flask. The digitonide need not be disturbed during the extraction. A few flakes that may come loose from the bottom of the flask quickly settle and the ether solution may be transferred by decantation or a dropping pipette without disturbing them.

Ten milliliters of water were added to the digitonide and gently boiled for two minutes. It was allowed to cool, and 20 ml. of acetone were added. After thorough mixing, the solution and suspended digitonide were transferred to a 50 ml. conical bottom centrifuge tube and the flask washed once with a little acetone. It is not necessary to remove all the digitonide from the flask.

The tube was then centrifuged at about 1500 or 2000 r.p.m. for five or ten minutes and the supernatant solution carefully decanted. A few milliliters of methyl alcohol were boiled in the flask in which the digitonide had been prepared and transferred to the centrifuge tube where they were again boiled. The hot solution was finally transferred to an oxidation flask. The precipitation flask and tube were washed twice with boiling methyl alcohol, and the washings added to the oxidation flask. The cholesterol was oxidized according to Boyd.[12]

Determination of Bound Cholesterol and Neutral Fat Fatty Acids.—The petroleum ether extracts of the free cholesterol digitonide contained bound cholesterol and neutral fat. The petroleum ether was evaporated and the lipid dissolved in 5 ml. of alcohol and saponified on the steam bath with 0.1 ml. of 50 per cent potassium hydroxide for one hour. When the alcohol had evaporated until no odor remained, the soaps were acidified with 1 ml. of 20 per cent phosphoric acid. The acid solution was heated on the steam bath for a few minutes and the lipid extracted several times with boiling petroleum ether. The ether extracts were transferred to a 50 ml. Erlenmeyer flask, and 2 or 3 ml. of digitonin solution were added. The flask was placed on the steam bath until dry. The fatty acids were extracted from the digitonide in the same manner as had been the fatty acid esters from the free cholesterol digitonide, and transferred to an oxidation flask. The bound cholesterol digitonide was treated exactly as the free.

Determination of Conjugated Lipids.—In the first series of analyses the conjugated lipids were fractionated into lecithin, cephalin, sphingomyelin, and cerebrosides in the following manner:

The acetone insoluble material, which contained the cerebrosides and the strontium chloride salts of the phospholipids, was dissolved in 0.2 ml. of chloroform, and 0.2 ml. of ammoniated methyl alcohol was added to decompose the the salt. The ammoniated methyl alcohol was prepared by dissolving 15 ml. of 29 per cent ammonium hydroxide in 86 ml. of methyl alcohol. This makes, approximately, a 5 per cent solution of ammonia.

The ammoniated solution was allowed to remain for a short time at room temperature. The chloroform, alcohol, and ammonia were then drawn off with reduced pressure. Bumping was prevented by holding the tube partially on its side and rotating it.

The dried mixture was then dissolved in a minimum volume of chloroform (not more than 0.2 ml.), and about 5 ml. of petroleum ether were added. Sphingomyelin and cerebrosides precipitated, while the unsaturated lipids, lecithin, and cephalin, remained in the solution. The tubes were centrifuged at about 2,000 r.p.m. until the supernatant solution was clear. The petroleum ether solution was transferred into another centrifuge tube, and the centrifugate washed with 3 ml. of petroleum ether. The petroleum ether solution of the lecithin and cephalin was evaporated on the steam bath while the suspended sphingomyelin and cerebrosides were being centrifuged from the petroleum ether washings. This washing was then added to the lecithin and cephalin and boiled to about 0.2 ml.

Five milliliters of absolute alcohol were added to this petroleum ether solution and the cephalin precipitated. After centrifuging, the supernatant solution was decanted into a 50 ml. Erlenmeyer flask. The cephalin was washed once with 3 ml. of absolute alcohol and the washings (separated by centrifuging) added to the lecithin solution. The cephalin was transferred to another 50 ml. Erlenmeyer flask by means of chloroform.

The sphingomyelin-cerebroside mixture was then extracted twice with 3 ml. portions of cold pyridine. This solvent dissolves the cerebrosides but not the sphingomyelin.[13] The washings were transferred to a 50 ml. Erlenmeyer flask. The sphingomyelin was dissolved in chloroform and also transferred to a 50 ml. Erlenmeyer flask.

The solutions were evaporated to dryness and saponified in 95 per cent alcohol, with 0.1 ml. of saturated potassium hydroxide. The extracted fatty acids were determined by oxidation.

In the second series of analyses, the petroleum ether soluble and insoluble fractions were not subdivided, but each was transferred to a 50 ml. Erlenmeyer flask and saponified. The fatty acids were made up to volume with petroleum ether in a 25 ml. volumetric flask, and an aliquot removed for oxidation.

Calculations.—The fatty acids of lecithin and cephalin were calculated as being oleic, and those of sphingomyelin and the cerebrosides as lignoceric. To calculate the weight of each of the conjugated lipids, the weight of its constituted acid was multiplied by the following factors:

Lecithin	1.39	Sphingomyelin	2.14
Cephalin	1.32	Kerasin	2.16

In the last set of analyses, when the lipids were divided into only the saturated and unsaturated fractions, the factor 2.15 was used for the former and 1.39 for the latter.

Neutral fat was calculated by multiplying the number of grams of neutral fat fatty acids, figured as oleic, by the factor 1.04.

Free and bound cholesterol were calculated by multiplying the number of milliliters of 0.1 N potassium permanganate required to oxidize the digitonide by the factor 0.0976.

Results.—Tables I, II, and III show the results of analyses of normal and degenerated nerves.

TABLE I

NERVE ANALYSES IN WHICH THE CONJUGATED LIPIDS WERE FRACTIONATED*

RAT	NEUTRAL FAT	FREE CHOLESTEROL	BOUND CHOLESTEROL	LECITHIN	CEPHALIN	SPHINGOMYELIN	KERASIN
Normal	0.70	3.12	0.159	6.34	2.86	1.62	5.93
Normal	1.53	2.92	0.137	6.47	2.80	1.32	5.60
Normal	0.98	2.41	0.148	----	----	----	----
Normal	1.06	3.50	0.190	6.55	4.98	1.62	4.54
Normal	2.48	3.47	----	8.04	3.36	1.70	5.52
Mean	1.35	3.08	0.153	6.85	3.50	1.56	5.42
First Group, Degeneration of Avitaminosis A							
1116	0.81	3.50	0.119	5.94	4.76	0.38	3.08
1117	----	3.89	----	----	4.34	1.09	3.17
1084	0.30	2.59	0.121	5.60	5.99	1.85	5.55
1085	0.41	2.63	0.074	4.86	3.56	0.77	3.26
1093	1.86	3.16	0.180	----	4.14	1.68	2.79
1103	2.01	3.20	0.306	6.99	2.29	1.99	2.06
Mean	1.08	3.42	0.160	5.85	4.18	1.29	3.32
Per cent change	-20.0	+11.0	+4.57	-14.6	+19.4	-17.3	-38.7
Second Group, Degeneration of Avitaminosis A							
E4477	2.62	2.53	0.331	6.45	3.45	1.93	----
E4452	2.94	2.45	----	6.04	4.61	1.09	2.32
E4455	3.45	2.41	0.104	5.06	2.05	1.44	3.15
E4476	1.71	2.36	0.048	5.61	1.23	0.54	3.32
E4481	2.60	2.44	0.171	7.83	3.12	0.86	4.84
Mean	2.66	2.44	0.163	6.20	2.90	1.17	3.41
Per cent change	+97.0	-20.8	+6.54	-9.49	-17.1	-25.0	-37.0

*Figures represent per cent of moist weight.

TABLE II

NERVE ANALYSES IN WHICH CONJUGATED LIPIDS WERE FRACTIONATED INTO SATURATED AND UNSATURATED GROUPS ONLY

RAT	NEUTRAL FAT	BOUND CHOLESTEROL	FREE CHOLESTEROL	SATURATED CONJUGATED LIPID	UNSATURATED CONJUGATED LIPID
Normal	0.60	----	----	4.60	11.4
Normal	0.50	----	----	5.80	10.6
Normal	----	----	----	5.07	11.1
Normal	----	----	----	5.20	11.1
Normal	0.79	0.18	3.73	6.17	10.5
Normal	1.69	1.16	3.56	6.02	----
Normal	2.18	0.21	2.86	5.40	12.2
Normal	1.67	0.20	2.73	5.68	10.6
Mean	1.22	0.19	3.22	5.49	11.1
Degeneration of Avitaminosis A					
1121	1.19	0.30	2.55	4.45	7.84
1123	0.99	0.24	3.30	4.19	12.40
1124	0.69	----	2.48	3.93	7.84
1125	0.98	0.32	----	3.57	13.40
1129	0.48	0.31	2.73	4.44	11.00
Mean	0.87	0.29	2.77	4.15	10.34
Per cent change	+28.7	+0.53	-14.0	-24.4	-7.20

TABLE III

A COMPARISON OF THE NORMAL NERVE MATTER GIVEN IN THE LITERATURE WITH THAT DETERMINED BY THE AUTHOR

INVESTIGATOR	CHOLESTEROL		PHOSPHOLIPID			CEREBROSIDE	TOTAL LIPID
Smith and Mair[23]		4.6		7.6		4.1	19.7
				5.65			18.8
				7.6			20.5
Koch and Koch[24]		2.00		5.39		3.24	19.5
		1.98		6.00			17.2
Koch and Voeglin[22]		3.34		8.52		3.36	18.4*
Jordan, Randall, and Bloor[14]		1.36		4.40		1.73	15.8†
Mott and Halliburton[16]				9.49			
			Lecithin	Cephalin	Sphingomyelin		
Koch[25]		4.86	5.19	3.49		4.57	19.5
	Free	Bound					
Verzár, Arvay, and Kokas[26]	4.08	0.66					
	1.25	0.15					
Gassner[27]		0.31					
		0.1					
Reiser	3.08	0.15	6.85	3.50	1.56	5.32	21.9‡
	3.22	0.19					
			11.1		5.49		

*Includes 3.49 per cent of sulfatides.
†Includes 8.35 per cent triglycerides.
‡Includes 1.21 per cent triglycerides.

DISCUSSION

The results of the analyses of normal nerves agree closely with the figures in the literature and are compared with those figures in Table III. An exception to this close agreement is the analyses of Jordan, Randall, and Bloor.[14] These authors obtained values higher in triglycerides and lower in all other lipids. Perhaps this is because they used methods intended for other kinds of tissues which contain very different proportions of the lipids. The other analyses reported were made by the use of methods especially designed for nerve material.

The evidence of the analyses presented above does not support the reasoning of Setterfield and Sutton[15] that the conjugated lipids are replaced by neutral fat in nerve degeneration due to vitamin A deficiency. There appears to be a significant increase in neutral fat in the E group of animals in the first series of analyses. However, it must be borne in mind that this fraction may not be nerve material at all but fat from connective tissue which may have adhered to the nerve during its excision. The animals of the E group were unusually fat for vitamin A deficient animals and one or two per cent more fat may have adhered to these nerves. It is also possible that this fraction was not neutral fat but unsaturated cerebrosides soluble in acetone.

The changes in free and esterified cholesterol cannot be considered as significant. Even though in the second series the cholesterol ester increased 50 per cent and the per cent deviation was very small, the total amount is so low that a larger series of determinations would have to be made before any significance could be attached to the increase.

The changes in the conjugated lipids present an interesting study. In all analyses, the petroleum ether insoluble lipids, which are considered to be sphingomyelin and kerasin, decreased considerably. Yet the petroleum ether soluble

phosphatides showed no significant decrease, but did, in one series, increase as much as 19 per cent.

The decrease of phosphatides and their replacement with neutral fat cannot, therefore, account for the disappearance of the anisotropic material in these degenerating nerves. This conclusion can be supported by evidence from the literature. Thus, Mott and Halliburton[16] and May[17] were able to find no decrease in the lipid phosphorus of the sciatic nerve during the first seven days of Wallerian degeneration. Yet Setterfield and Sutton[15] have shown that about half of the anisotropic material has disappeared during this time. Mott and Halliburton also noticed a positive Marchi reaction in degenerating nerves before there was any change in phosphorus content.

It is possible that the paradoxes can be explained on the basis that in a normal functioning nerve, the conjugated lipid material is in an organized form, perhaps in some conjugation with protein. It is this organized material that is birefringent and fails to stain with the fat stains. The first stage of nerve degeneration may then be postulated as being a breakdown of this organization. The lipids are still in the nerve, but are nonfunctional, do not rotate polarized light, and can be stained. The fact that the organized fats of living tissues do not stain is well known and has been emphasized by Wells.[18]

The next step is the removal of the lipids from the nerve. In accord with the evidence presented in this paper, the stable saturated lipids may be converted to the more labile unsaturated ones and thus be removed.

Evidence that phospholipid is increased in degenerating nerve material has been presented in the literature. Pighini and Fontesesi[19] have shown that phospholipids increase during the aseptic autolysis of the spinal cord of a dog. Backlin[9] found a decrease in cerebrosides and a possible increase in the phospholipids of brain during autolysis, and Singer[20] found an increase in phospholipid content of the frontal lobes in progressive paralysis. Koch,[21] however, found a decrease in phospholipid in the central nervous system during general paralysis. Koch and Voeglin[22] observed a much greater decrease in the cerebrosides than in the phospholipids in the spinal cord in pellagra.

SUMMARY AND CONCLUSIONS

1. A new micromethod for the partition of the lipids in nerve material has been developed.

2. An acetone-soluble material, not cholesterol, was found in the sciatic nerves of albino rats.

3. There is a decrease in the saturated conjugated lipids, calculated as sphingomyelin and kerasin, during nerve degeneration due to lack of vitamin A.

4. There is no decrease in lecithin and cephalin during nerve degeneration due to lack of vitamin A.

5. Bound cholesterol was found in nerve tissues.

6. Free cholesterol does not change significantly during nerve degeneration due to lack of vitamin A.

7. The significance of these findings was discussed.

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METASTATIC BONE CARCINOMATOSIS ASSOCIATED WITH PARATHYROID HYPERPLASIA AND WITH SYMPTOMS OF HYPERPARATHYROIDISM*

With Blood Calcium and Phosphorus Studies Before and After Extirpation of a Hyperplastic Parathyroid Gland

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THE role of the parathyroid glands in calcium metabolism is now well recognized. The investigations of Hanson[1] and Collip[2] have demonstrated that these glands produce a hormone whose function is to regulate the calcium level of the blood. Injection of this hormone in animals and in the human being produces a rise in the level of the serum calcium.† Extirpation of the parathyroid glands produces a state of tetany with a fall of serum calcium. In certain pathologic states alterations of the parathyroid glands sometimes occur. In osteitis fibrosa cystica, or hyperparathyroidism, in which condition there is a constant hypercalcemia, an adenoma of a parathyroid gland is usually found. This adenoma is considered to be the primary and etiologic factor in the production of the disease. Removal of the adenoma is followed by clinical improvement, with a fall of the blood calcium to normal levels. The disease has been produced experimentally by the prolonged administration of parathyroid hormone.[3]

The syndrome of hyperparathyroidism is now a well-recognized clinical entity. The diagnosis is based upon a history of renal calculi and arthritic pains; the presence of hypercalcemia, hypophosphatemia, and an increased excretion of calcium in the urine; the finding of muscular hypotonia, widespread rarefaction of the bones, and a parathyroid tumor. Of these, the hypercalcemia and the changes in the bones are the most characteristic findings. The presence of a high serum calcium in a patient with muscular hypotonia, or arthritic pains, should lead one to suspect hyperparathyroidism.

Although the x-ray of the bones and the blood findings are characteristic, confirmation of the diagnosis is sometimes very difficult, as evidenced by reports in the literature of cases operated upon several times in an attempt to find the adenoma. The difficulties encountered in the differential diagnosis are exemplified by a case reported by Mason and Warren.[4] Their patient presented widespread bony changes, hypercalcemia, and a negative calcium balance. Al-

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†The rise is either the result of a direct action of the hormone on the calcium, or, as Albright[11] suggests, the hormone first causes a fall in the blood phosphorus followed by a secondary rise in the calcium.

though the x-ray examination of the bones suggested metastatic carcinoma, a search was made for a parathyroid adenoma. At the operation a metastatic carcinoma to the thyroid and parathyroid glands was found which originated from a mammary cancer removed five years before. The hypercalcemia, they state, was either due to an associated hyperparathyroidism, or was an accompaniment of widespread bone metastasis.

In rickets, osteomalacia, and osteogenesis imperfecta, in which conditions there is a low level of serum calcium, hyperplasia of the parathyroid is often found. This hyperplasia is considered to be secondary in nature.

In 1923 Klemperer[5] reported a case of breast carcinoma with metastases to the bones associated with parathyroid hyperplasia. Barr and Bulger[6] in 1930 reported a case of multiple myeloma with parathyroid hyperplasia, hypercalcemia, a negative calcium balance, increased urinary calcium and metastatic calcification in the lungs, stomach, and kidneys. They considered the hyperplasia as secondary to the bone changes, and the hypercalcemia as a manifestation of increased function of the parathyroid glands.

Thus we find two instances in the literature of parathyroid hyperplasia occurring secondary to widespread bone destruction: one case, that of multiple myeloma, was associated with hypercalcemia and other symptoms of hyperparathyroidism; in the other case of metastatic carcinoma there were no calcium studies recorded.

A case is presented here with definite bone metastases associated with parathyroid hyperplasia, and with symptoms of hyperparathyroidism. Blood calcium studies were made before and after the extirpation of a hyperplastic parathyroid gland.

CASE REPORT

Clinical History: Patient A. G., a man aged 58 years, was admitted to the Greenville Hospital on Nov. 5, 1935, complaining of pain in the right shoulder and right arm, right hip, and right leg. The family history was insignificant. His previous history was negative, except for "flu" in 1918. There was no history of any venereal infection. The patient was in good health until about two months previously, when he began to have pain in the right shoulder and right arm. He consulted a physician who informed him that he had "neuritis." About one month later he began to have pain in the right hip radiating down the right leg. His pain gradually became more severe, and he lost about 10 pounds in weight. There were no gastrointestinal, respiratory, nor genitourinary symptoms.

Physical examination revealed a rather thin, somewhat emaciated adult. The muscles were rather flabby. There was slight limitation of motion about the right shoulder joint. The thyroid gland was not enlarged. There were no masses nor enlarged glands felt in the neck. The heart and lungs were normal. Examination of the abdomen did not reveal anything abnormal. The prostate gland was not enlarged. There were no nodules in the axillae nor in the supraclavicular regions. The reflexes were normal. The blood pressure was 120/80.

The laboratory examination revealed the following: The urine was negative for sugar, albumin, and Bence-Jones protein. There were no red and white blood cells. An occasional hyaline cast was seen. The blood count showed a hemoglobin of 80 per cent, red blood cells 4,580,000, white blood cells 8,800, polymorphonuclear cells 70 per cent, small lymphocytes 21 per cent, monocytes 6 per cent, and eosinophiles 3 per cent. The blood chemistry showed nonprotein nitrogen 32 mg., urea nitrogen 16 mg., calcium 14.2 mg., phosphorus 2.4 mg. per 100 c.c. of blood. The phenolphthalein excretion was 60 per cent in two hours. The Wassermann and Kahn tests were negative.

X-ray studies of the genitourinary tract, chest, and gastrointestinal tract gave negative results. X-ray examination of the right humerus (Fig. 1) showed a large multilocular cyst in the upper third of the shaft with expansion and thinning of the cortex. There was some rarefaction of the bone below the cyst. X-ray of the skull (Fig. 2) showed multiple areas of rarefaction. These areas varied in size from ⅛ inch to 1 inch in diameter. The surrounding structure showed no changes. There was some rarefaction in the bones of the pelvis and right femur and in both fibulae. These findings suggested bone metastases, although rarefaction due to hyperparathyroidism could not be excluded.

On November 9 the patient was placed on a low calcium diet, and after three days, the calcium and phosphorus in the urine and stool were determined for the following three days. The results of these studies are shown in Tables I, II, and III.

TABLE I

CALCIUM AND PHOSPHORUS INTAKE

DATE	CALCIUM	PHOSPHORUS
	mg.	mg.
November 9	106	146
November 10	110	410
November 11	120	150
November 12	110	162
November 13	118	360
November 14	102	126

TABLE II

CALCIUM INTAKE AND OUTPUT

DATE	CALCIUM INTAKE	CALCIUM OUTPUT	
		URINE	STOOL
	mg.	mg.	mg.
November 12	110	106	32.4
November 13	118	124	36.6
November 14	102	146	26.6
	330	376	95.6

Total calcium intake	330.0 mg.
Total calcium output	471.6 mg.
Negative balance	141.6 mg.

TABLE III

SERUM CALCIUM AND PHOSPHORUS

DATE	SERUM CALCIUM	SERUM PHOSPHORUS
	mg.	mg.
Nov. 6, 1935	14.2	2.4
Nov. 8, 1935	13.8	2.6
Nov. 10, 1935	14.0	2.2
Nov. 12, 1935	14.6	2.0
Nov. 13, 1935	16.2	1.6
Nov. 14, 1935	14.2	2.0
Nov. 15, 1935	14.8	1.8
Operation Nov. 18, 1935		
Nov. 22, 1935	14.0	2.4
Nov. 27, 1935	13.2	3.0
Dec. 16, 1935	12.6	3.2
Dec. 23, 1935	11.4	2.4
Jan. 8, 1936	12.6	3.2
Jan. 27, 1936	13.2	2.2
Feb. 4, 1936	13.0	2.6
May 6, 1936	13.4	2.8

It was evident from these studies that the patient had a persistent hypercalcemia. The blood phosphorus was low. The normal relationship of a higher calcium excretion in the feces was reversed, so that there was an increased ratio of calcium in the urine to the total amount of calcium excreted. The patient was also in a negative calcium balance. Because of these findings, an exploration for a parathyroid tumor was deemed warranted.

Fig. 1.—Roentgen ray of right humerus, showing multilocular cyst formation.

Operation was performed by Dr. H. Jaffe on Nov. 18, 1935. The following is the report: "Under general anaesthesia of gas and ether, a typical collar incision was made as for thyroid. In the region of the right upper pole somewhat posteriorly a rounded nodule, the size of a hazel nut, was felt. The right lobe was grasped with a Lahey goiter forceps and the lobe completely dislocated, thus allowing access to the posterior surface. In the region of the inferior pole what appeared to be an enlarged parathyroid gland was found. This was excised as well as the nodule within the upper pole of the lobe. The small mass of tissue

measured 9 by 4 by 5 mm. and the larger 1.8 cm. in diameter. The same procedure was followed on the left side, but no masses were found. The glands were replaced and a careful search below the region of the isthmus and in the upper thoracic space was made. No other abnormally situated enlarged parathyroid was found."

Fig. 2.—Lateral view of skull, showing areas of rarefaction.

The following is the pathologic report of the excised specimens (Dr. W. Antopol):

Gross Examination: (a) A small nodule of tissue, measuring 7 by 2 by 4 mm.,* appears to be encapsulated. On cut section this shows a brownish homogeneous color. (b) A mass of tissue, about 1.5 cm. in diameter, which on cut sections shows extensive colloid areas as well as small areas of hemorrhage.

Microscopic Examination (Fig. 3): (a) Reveals the smaller nodule to be composed of polyhedral cells with abundant clear cytoplasm and a single round nucleus which contains considerable chromatin. These cells are arranged in the form of nests and alveoli. No glandular or cystic elements are noted. (b) Reveals thyroid tissue with a considerable amount of colloid in distended alveoli, areas of hemorrhage, and fibrosis.

Diagnosis: Hyperplastic parathyroid. The patient stood the operation very well. The next day he was quite comfortable. For the remaining days in the hospital he complained of pain in the right shoulder and right leg. His blood calcium was still high. He refused to stay in the hospital any longer and was discharged on Nov. 27, 1935.

Examination of the patient on Dec. 16, 1935, at the office showed that he was gradually getting worse. The pain in the right shoulder and right leg was very severe. His appetite was fair, but he felt weaker. His blood calcium was 12.4 mg., and his blood phosphorus was 2.6 mg.

It was felt that a re-exploration might reveal another parathyroid enlargement which when removed would result in improvement. On Jan. 8, 1936, he was admitted to the Medical Center. An operation was performed by Dr. R. Lobban on Jan. 10, 1936. The neck and upper thoracic space were thoroughly re-explored, but no other parathyroid enlargement was discovered.

*The discrepancy in the measurements is due to the shrinkage of the tissue in the formalin.

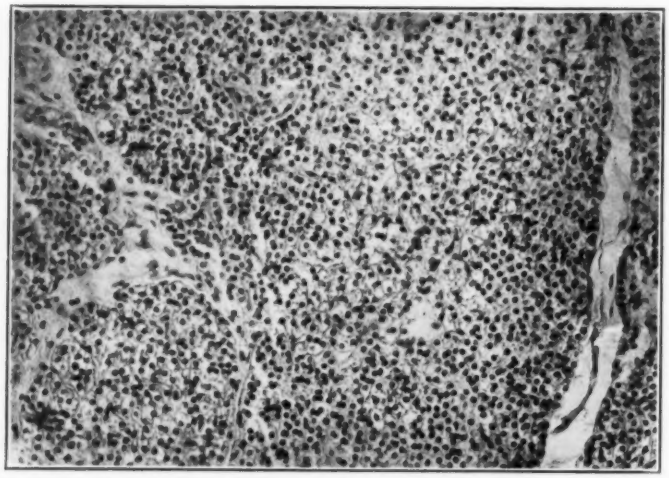

Fig. 3.—Microscopic appearance of the hyperplastic parathyroid gland.

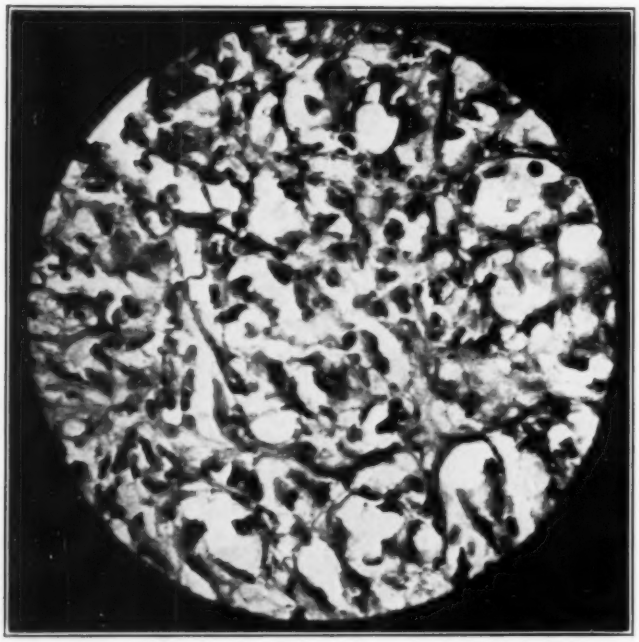

Fig. 4.—Microscopic appearance of a biopsy of the right humerus, showing metastatic carcinoma.

A biopsy of the right humerus was taken and following is the pathologic report. (Dr. N. Alter):

Microscopic section of the bone marrow (Fig. 4) shows no characteristic tissue elements. These have been replaced by a neoplastic growth which consists of large, pale, somewhat elongated, epithelial cells. There is great variety in shape and size of cells and nuclei. The epithelial cells form solid cords and masses. There is no evidence of keratinization, but there are areas of necrosis where polynuclears are present; otherwise, there is little fibrous stroma with lymphocytic reaction.

Microscopical Diagnosis: Metastatic carcinoma of bone marrow.

The patient was discharged on Jan. 19, 1936. Examination of the patient on May 6, 1936, at the office showed that the patient had lost a great deal of weight and felt very weak. He complained of a cough which began three weeks before. There was moderate dullness in the right infraclavicular region, with bronchovesicular breathing. The pulse was weak and rapid.

An x-ray of the chest revealed an irregular dense shadow, 2¼ inches in length and 1 inch in width, beginning at the right hilus and extending upward toward the right clavicle. Its margin was irregular due to small striae spreading out into the adjacent tissue. The appearance of the shadow was that of an expanding lesion.

Examination of the sputum was negative for tubercle bacilli. He refused hospitalization and bronchoscopic examination. It was believed that the lesion was a bronchiogenic carcinoma, with metastases to the bones.

On May 21, 1936, the patient had a profuse hemoptysis. The signs in the chest were more marked. He was confined to bed, with repeated attacks of hemoptysis. He gradually became weaker, and died on Nov. 5, 1936, at his home. Permission for autopsy was not granted.

DISCUSSION

The case presents many points of interest. It has generally been accepted that hypercalcemia associated with rarefaction of the bones is pathognomonic of hyperparathyroidism. The parathyroid hyperplasia or adenoma found in this condition is the primary cause, and the clinical symptoms are the result of increased parathyroid function. In the case presented here the hypercalcemia was found to be associated with metastatic carcinoma of the bones and with parathyroid hyperplasia. A search through the literature fails to reveal a similar case with blood calcium studies following the removal of a hyperplastic parathyroid.

The question arises whether the hypercalcemia was the result of hyperfunction of the parathyroid, or whether it was due to excessive bone destruction. Barr and Bulger believe that in their case of multiple myeloma, the hypercalcemia may have reflected increased function of the parathyroid glands.

It will be noted that in our case the removal of the hyperplastic parathyroid gland did not reduce the serum calcium to normal, but the calcium level was somewhat lower than before the operation. It should also be noted that the lowering of the serum calcium was observed ten days after operation and lasted for about ten weeks, after which there was a slight rise. It seems reasonable to assume that both the bone destruction and the hyperplasia may have been factors in producing the hypercalcemia, and, as Barr and Bulger suggest, the bone changes may have stimulated the parathyroid which in turn aggravated the condition. It must be conceded, however, that the failure to find another pathologic parathyroid in the second attempt does not eliminate the possibility of an abnormally situated one, which would account for the persistence of the hypercalcemia.

It would further be of interest to discuss the nature of the parathyroid hyperplasia. The finding of a parathyroid tumor in a case of osteitis fibrosa cystica was first reported by Askanazy[7] in 1904. Erdheim[8] later noted the frequent occurrence of parathyroid hypertrophy in rickets, and concluded that the hypertrophy represented an attempt at compensation for the calcium changes in the blood. This theory was generally accepted and applied also to osteitis fibrosa

cystica. Hoffheinz[9] later pointed out that in practically all cases showing parathyroid hyperplasia, only one gland was found involved, which fact is a strong argument against the theory of compensation.

In 1925 Mandl[10] transplanted four normal human parathyroid glands from a patient dying as a result of an accident into the abdominal wall of a young man with osteitis fibrosa cystica. The patient instead of getting better became worse. A month later Mandl explored the neck and found one of the parathyroid glands to be the site of an adenoma. The gland was removed, followed by striking symptomatic improvement. He thus showed that the changes in the parathyroid gland in osteitis fibrosa cystica were the cause rather than the result of the alteration in the bones.

Klemperer suggests that in his case of bone carcinomatosis, the hyperplasia might be considered as a fruitless attempt of the body to compensate for the loss of calcium, and that it might be conceived as a hypofunctioning rather than a hyperfunctioning gland.

Barr and Bulger believe that in their case of multiple myeloma, the hyperplasia of the parathyroids is secondary to the bone changes and that the hypercalcemia is a manifestation of hyperfunction of the glands.

It is obvious that in our case, also, the hyperplasia was secondary to the bone changes. Since the removal of the gland resulted in somewhat lower level of the blood calcium, it is reasonable to conclude that the gland showed evidence of hyperfunction. This would support the theory of Erdheim of compensatory hyperplasia.

We thus find compensatory hyperplasia in conditions associated with low serum calcium, such as rickets and osteomalacia, and also in states where the serum calcium is high. This apparent paradox is difficult to explain. Further studies of parathyroid physiology might throw more light on this subject.

SUMMARY

A case is presented with metastatic carcinoma associated with parathyroid hyperplasia and with symptoms of hyperparathyroidism, such as hypercalcemia, a negative calcium balance, and increased excretion of calcium in the urine. Removal of the gland resulted in a somewhat lower but not normal level of the serum calcium. The nature of hypercalcemia and the hyperplasia is discussed.

I wish to thank Dr. H. Jaffe, Dr. R. Lobban, Dr. Wm. Antopol, and Dr. N. Alter for their invaluable aid in this case.

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THE REVERSAL OF KIDNEY VOLUME RESPONSE ACCOMPANYING ABOLITION OF THE CENTRAL NERVOUS SYSTEM*

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COHNHEIM and Roy[5] (1883) first demonstrated the vasoconstriction produced in the kidney by stimulation of the splanchnic nerve. Bradford[3] (1889) repeated this observation and also showed that stimulation of the nerve at a low frequency (about 1 per second) produces dilatation rather than constriction of the kidney. Bardier and Frenkel[1] (1899) studied the vasoconstrictor action of adrenal extract on the kidney, and, in two instances, observed a slight increase in volume.

One of us (E. C. M.), while working in the laboratory of Dr. D. E. Jackson (1920), observed a marked increase in kidney volume following the destruction of the central nervous system and the administration of epinephrine. Since the authors have been unable to find any reference in the literature to studies on the relationship between the destruction of the central nervous system and kidney volume response, we have repeated our observations and attempted to find an explanation for the reversal of kidney volume response under such conditions.

EXPERIMENTAL PROCEDURE

All of the experiments were performed on healthy dogs, ranging in weight from 7 to 20 kg. They were anesthetized either with ether or with sodium pentobarbital (nembutal, Abbott), ether being used in the majority of the experiments. If nembutal was used, it was injected intraperitoneally in doses of 1 grain per 5 pounds body weight, augmented by intravenous doses of one-tenth the original when required.

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Received for publication, September 22, 1938.

Blood pressure was recorded from the right carotid artery, using a mercury manometer attached by rubber tubing to a glass arterial cannula. The recording system was filled with 20 per cent sodium citrate. The Jackson kidney and spleen oncometers and recording tambours were used to record kidney and spleen volume changes. The kidney oncometer was usually placed on the left kidney since the right lies somewhat higher in the abdomen than the left, its pedicle is shorter, and it is usually bound by a heavy renohepatic ligament which must be torn loose, producing severe trauma to the kidney, and frequently partial decapsulation or rupture of the inconstantly present blood vessels to the kidney or adrenal gland.

Injections of epinephrine were made directly into a 2 cm. section of the right femoral vein, exposed just below the inguinal ligament.

Destruction of the Central Nervous System.—The scalp is slit in the midline from just anterior to the occipital protuberance to about 2 cm. behind the eyes, whence the incision is carried laterally behind the eye for 3 or 4 cm. The temporalis muscle is cut through, and the area of origination is scraped clean. Using a Galt trephine, a hole is cut through the skull and the button of bone is removed. With the handle of a scalpel the brain is thoroughly macerated. This procedure liberates brain tissue extract, which is an efficient hemostatic, preventing death from hemorrhage. The ether bottle is quickly disconnected, since anesthesia is not required after destruction of the central nervous system, and either a bellows or an artificial respiration pump is attached in its place. The animal's head is returned to its original position, and the pithing wire is introduced through the foramen magnum. The pithing wire is made of a single strand of 16 gauge copper wire bent back on itself and twisted. It is passed down the spinal canal with an interrupted, twisting motion, thoroughly macerating the spinal cord. The wire is then withdrawn, pushing it backward and forward throughout the extent of the spinal canal, and the trephine hole is plugged with a gauze sponge. If pithing is complete, the blood pressure will fall to about 40 mm. Hg and maintain a constant level.

KIDNEY VOLUME CHANGES IN RESPONSE TO EPINEPHRINE

Normal Response.—In the normal animal the kidney volume reaction to intravenously administered epinephrine is entirely, or almost entirely, a reduction in volume. Fig. 1 is the record obtained from an unpithed dog, weighing 11.8 kg., anesthetized with ether. The top line is kidney volume, the middle line is blood pressure, the lowest is time in ten-second intervals and the base line for blood pressure. At the point marked (X), 0.3 c.c. of epinephrine at a concentration of 1:10,000 was injected intravenously. It will be observed that the kidney volume curve shows a marked fall during the time of the rapid rise in blood pressure, a period during which the constriction was maintained, corresponding to the maximal elevation of blood pressure, and a return to nearly normal volume. Inasmuch as the only contractile element of any importance in the kidney is the musculature of the blood vessel walls, it follows that any changes in kidney volume must be due to changes in the caliber of the vascular channels (Herrick, Essex, and Baldes, 1932[7]).

There is a deviation from this reaction which occurs occasionally. As shown in Fig. 2 there is sometimes a slight increase in the volume of the kidney accompanying a slight fall in systemic blood pressure before the principal fall in kidney volume and rise in blood pressure occur. Fig. 2 shows not only the curves of carotid blood pressure and kidney volume, but also the record of spleen volume as well. The preliminary fall in blood pressure and rise in kidney volume is probably due to active vasodilatation.

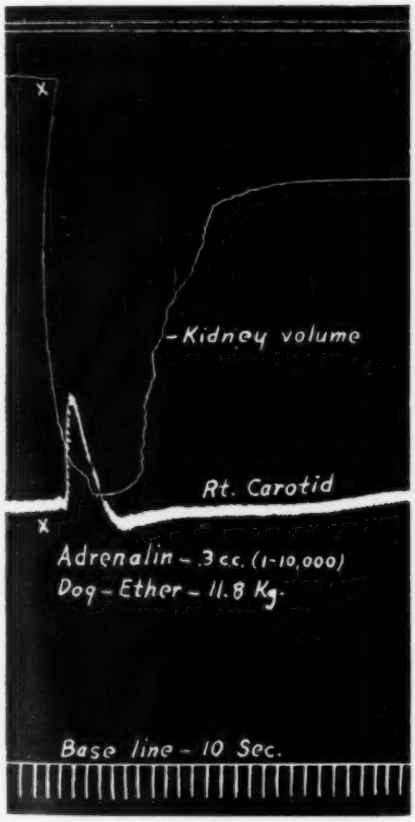

Fig. 1.

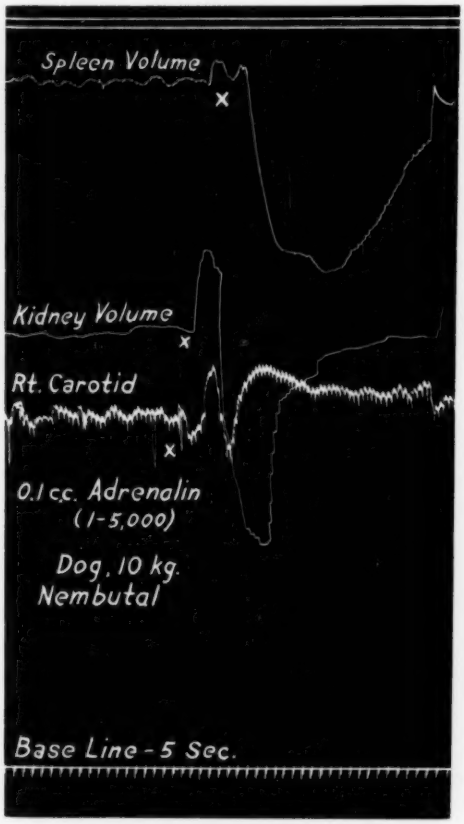

Fig. 2.

Kidney Volume Reaction in Pithed Animal.—The kidney volume reaction to intravenously administered epinephrine is altered by pithing, as is demonstrated in Fig. 3, a record obtained from a dog weighing 7 kg. The animal was operated upon under ether, the brain and spinal cord were pithed completely, and artificial respiration was instituted. The upper curve is kidney volume, the middle line carotid blood pressure, and the lowest the base line and time record. It will be observed that the blood pressure is much smoother than that in the unpithed animal and that the blood pressure reaction to 0.5 c.c. of epinephrine (1:10,000) is a precipitous rise, the maximum being approached more quickly than in the normal.

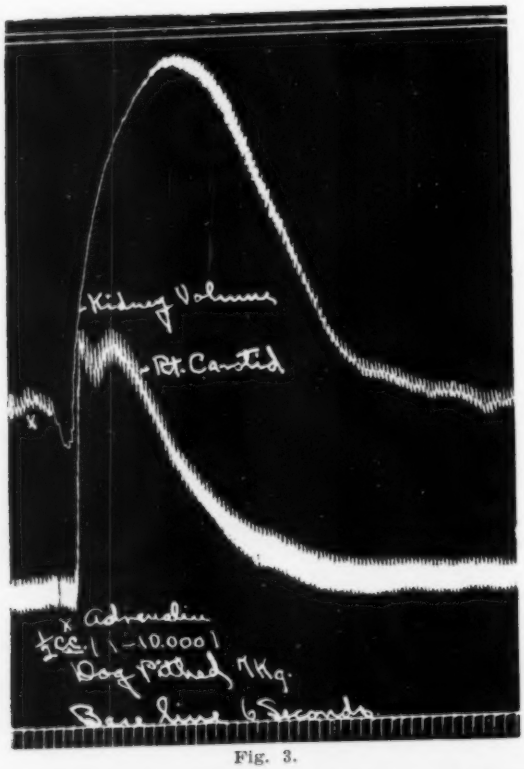
Kidney Volume
Rt. Carotid
x
x Adrenalin
1/2 cc. (1-10.000)
Dog pithed, 7 Kg.
Base line 6 Seconds

Fig. 3.

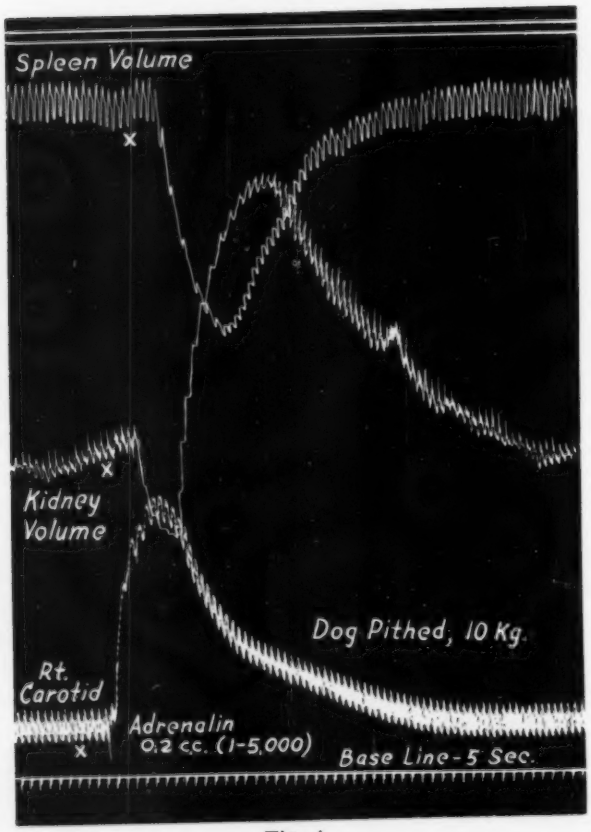
Spleen Volume
x
x
Kidney Volume
Dog Pithed, 10 Kg.
Rt. Carotid
x
Adrenalin
0.2 cc. (1-5,000)
Base Line - 5 Sec.

Fig. 4.

The kidney volume curve is characterized by, first, a fall of much smaller extent than the normal, a short period during which the constriction is maintained, and then an increase in volume to a level far above the normal. The ascending limb of the curve begins at about the time the blood pressure reaches its maximum. The final phase is a descent to normal, the kidney volume falling more rapidly than the blood pressure, so that the two reach basal levels at about the same time.

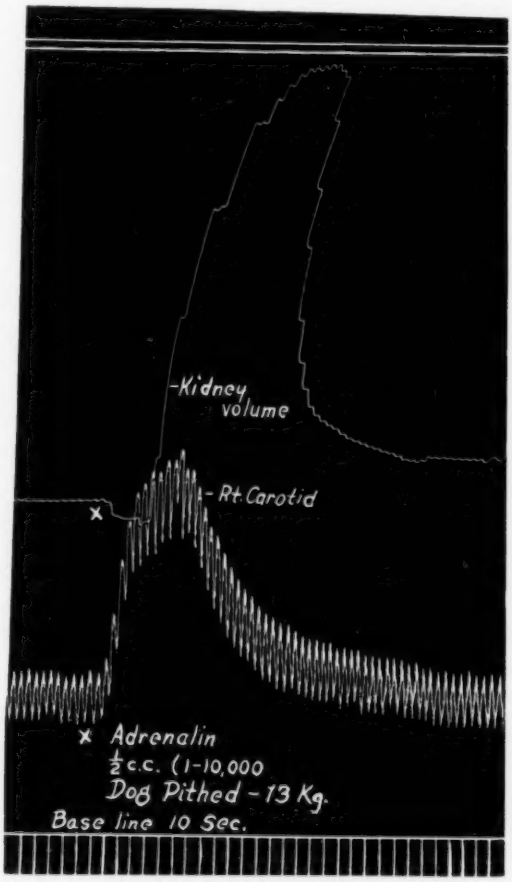

Fig. 5.

Fig. 4 is presented in order to illustrate the difference in response to adrenalin before pithing and after pithing. This tracing was obtained from the same animal as Fig. 2. It will be observed that the spleen volume decreased in both tracings, but that the kidney volume response was just the reverse of that observed in Fig. 2. It is probable that the various other organs do not respond as the kidney does, by dilatation after destruction of the central nervous system, for if such response were generalized, there would of necessity be a drop in blood pressure with such generalized organ dilatation.

Apparently the anesthetic used was without marked effect on the responses obtained, since Figs. 2 and 4 were obtained from an animal anesthetized with

sodium pentobarbital, and the remaining Figs. 1, 3, 5, and 6 were obtained from animals anesthetized with ether. While there may be some difference in the degree of response produced, the type of curve is approximately the same.

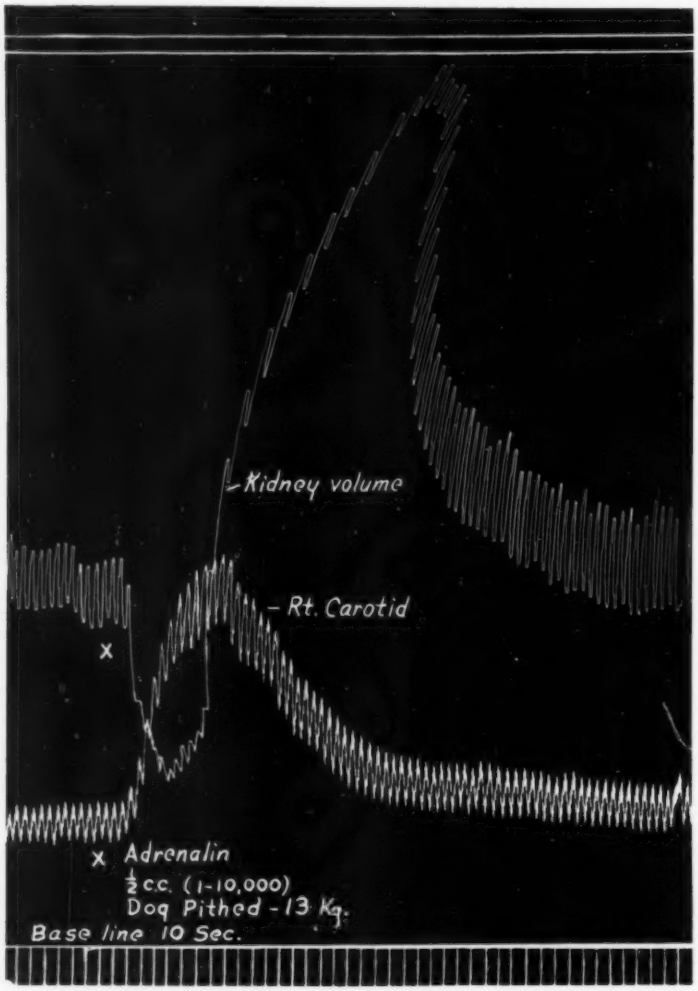

Fig. 6.

DEDUCTIONS

In searching for an explanation for this reversal of kidney volume response following destruction of the central nervous system, we are forced to consider the following:

First: The obstruction of the venous return from the kidney. It is evident that any mechanical obstruction of the renal veins, such as might be produced by faulty application of the kidney oncometer, would interfere with the return circulation, and thus cause an increase in the size of the kidney as a response to increased blood supply. This factor may be ruled out in our experiments, since analysis of our results show that (1) the return kidney volume to normal accom-

panies the blood pressure curve; (2) we have repeatedly obtained the same type of curve from the same animal, reproducing it as often as fifteen times; (3) animals, such as the one producing records (2) and (4), gave a decrease in kidney volume response before pithing and an increase following destruction of the central nervous system.

Second: Possible shocklike condition of the animal with an accompanying state of stimulation of the vasomotor system. If this explanation is offered, it must be considered as specific for the kidney, for it will be recalled that the spleen does not share the volume reversal after destruction of the central nervous system. Also, if all other organs responded as the kidney does, the blood pressure would not show the sudden and marked rise recorded. The best results are observed when the oncometer is speedily and easily attached and when pithing is done quickly; in other words, the best results are obtained with good, not faulty, technique.

Third: Direct stimulation of vasodilator nerve endings. Bardier and Frenkel[1] on two occasions observed vasodilation in the renal vessels after small doses of the extract of the adrenal medulla. Biberfeld[2] and Ogawa[8] confirmed these results, although Elliott[6] was unable to do so. Also, it has been repeatedly demonstrated that the kidney will respond to epinephrine or stimulation of the splanchnic nerve by dilatation after the administration of ergotoxine or ergotamine. The following two points suggest that the increase volume response of the kidney is not due to the stimulation of the vasodilator nerve endings: (1) The threshold of the vasodilator is low, responding to minute doses of epinephrine, the concentration used to demonstrate vasodilating action usually being 1:100,000 in doses of 0.1 to 3 c.c. Our experiments were performed using concentrations of 1:5,000 and 1:10,000 in doses ranging from 0.1 to 2 c.c. (2) Cannon and Lyman[4] found that epinephrine did not produce vasodilation when the blood pressure fell below a certain minimal level, which they placed at 50 mm. Hg, although it can be produced at such a blood pressure level if ergotoxine is administered.

Fourth: Manipulation of the kidney damaging the vasoconstrictors of the kidney. This possibility is ruled out by (1) the record obtained on a normal intact animal showing a decrease in kidney volume, while the same animal, without additional manipulation of the kidney, showed a reversal of kidney volume after destruction of the central nervous system; (2) the best results are obtained when the manipulation and kidney damage are the least.

Fifth: The possible destruction of vasoconstriction mechanisms low in the cord. The possibility of destruction of subsidiary vasoconstrictor centers in the cord must be considered. The destruction of these centers, which are possibly in balanced opposition to the vasodilators of the kidney, would permit adrenalin to act on the vasodilators unopposed. However, it is difficult to conceive how such a mechanism would involve the kidney and not the other organs, such as the spleen.

Sixth: Passive dilation produced by the increased blood pressure. In all cases the kidney responds in the pithed animal by an initial slight decrease in volume followed by a marked increase in volume, the maximum volume of the

kidney corresponding to maximum of the blood pressure. The two, kidney volume and blood pressure, then decrease simultaneously and reach the basal level together. This would be the sequence in passive dilatation of the kidney.

CONCLUSIONS

1. The kidney volume response to epinephrine injections is changed by the destruction of the central nervous system, the response being essentially a reversal of that observed in the intact animal.

2. The increase in kidney volume accompanies the increase in blood pressure; therefore, the response might be a passive dilatation of the kidney.

3. There is also a possibility that destruction of subsidiary vasoconstrictor centers in the cord allows epinephrine to stimulate the unopposed dilatating fibers of the kidney.

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A NEW APPARATUS FOR OXYGEN THERAPY*

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OXYGEN therapy is often lifesaving when properly administered. However, the initial cost of therapeutic equipment, the expense of operation, the discomforts to the patient as well as lack of safety, inconvenience, and many more factors, contribute to the all too frequent denial or improper administration of oxygen therapy. In order to overcome these and other objections, various types of apparatus have been developed and tested on the wards of the Los Angeles County General Hospital. The equipment described below has been the most acceptable and has been in use since December, 1935. Satisfactory therapeutic results are obtained with a flow of 4 to 8 liters of oxygen per minute. This compares favorably with flow rates used with tents, nasal catheters, and other economical methods now in use. In the following discussion data will be introduced showing the effectiveness of this apparatus

*From the Pathological Laboratory and the Service of Dr. Roy Thomas of the Los Angeles County General Hospital, Los Angeles.

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in increasing the alveolar oxygen tension of normal individuals. The clinical effectiveness of this apparatus will be included in a later report. It may be noted here that the results from the study of normal individuals have been amply substantiated on the wards of the Los Angeles County General Hospital, where approximately one million cubic feet of oxygen are given yearly by this method.

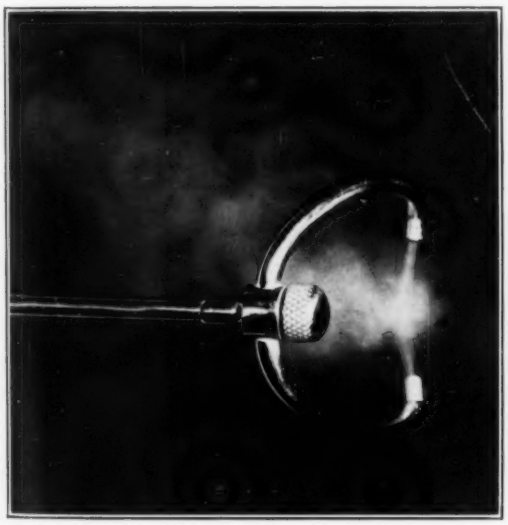

Fig. 1.

Fig. 2.

APPARATUS

The apparatus is an inhaler which is essentially a tubular Y constructed of metal or durable material. Gas issues from the curved arms of the Y in such directions that the streams meet and neutralize each other's velocities with the exception of a slightly resultant vector directed toward the center of the patient's upper lip. A high concentration of oxygen is held in the region most effective for inhalation, but without the irritating effect of a direct jet against the skin. This principle is shown in Fig. 1, using smoke in place of oxygen. The ordinary form of the inhaler has the stem portion as shown in Fig. 2,* and is attached to the patient's forehead by means of adhesive tape (see Fig. 3), head band, or spectacle frame.

To increase the efficiency, a mask of cellulose acetate or similar material, made preferably in two sections, is pivotly mounted by means of eyelets on the ends of the fork (see Fig. 2). The mask in two sections allows the patient to have access to his mouth without removal of the equipment.

*The mask in this figure is the large mask referred to later in the table.

TABLE I

SUMMARY OF RESULTS

SUBJECT	SEX	WEIGHT	HEIGHT	AGE	NORMAL ALVEOLAR AIR		RATE OF FLOW OF OXYGEN PER MINUTE										REMARKS
							2 LITERS		4 LITERS		6 LITERS		8 LITERS		10 LITERS		
							ALVEOLAR		ALVEOLAR		ALVEOLAR		ALVEOLAR		ALVEOLAR		
					% O_2	% CO_2	% O_2	% CO_2	% O_2	% CO_2	% O_2	% CO_2	% O_2	% CO_2	% O_2	% CO_2	
D. B. T.	M	200	6′1″	33	15.7	4.9	22.1	5.4	30.2	5.7	36.8	4.7	44.4	4.9	45.5	5.3	Small mask, closed
					15.1	5.4	21.4	5.4	30.0	4.8	34.1	5.3	38.8	5.3	47.1	5.0	Small mask, closed
					15.6	4.3	21.9	6.1	28.5	5.8	31.6	5.8	46.0	5.9	41.3	5.2	Large mask, closed
					16.3	4.4					31.7	5.8					
					13.1	6.4			24.0	5.8	29.7	5.1	25.0	5.6	30.3	5.6	Small mask, open
					13.1	5.5	20.1	5.7	26.8	5.5	32.9	5.5	38.5	5.9	45.8	6.2	Large mask, closed
					14.2	5.9	24.0	5.3	25.4	5.4	33.9	5.3	36.0	4.8	37.1	5.7	Large mask, closed
C. F. L.	M	150	5′10″	30			26.0	6.7	37.4	6.2	57.2	6.1	47.8	6.1	53.7	5.4	Small mask, closed (mouth breathing)
					13.0	6.8	21.4	6.5	35.2	7.1	46.4	6.8	58.8	6.4	59.7	6.2	Small mask, closed
					12.5	7.0			26.0	6.2	38.5	5.6	34.2	5.6	50.3	5.8	Small mask, open
							22.9	6.4	33.0	5.9	44.9	6.0	53.9	6.4	57.2	5.8	Large mask, closed
M. H.	F	120	5′8″	24			23.4	5.6	31.3	4.3	54.0	5.1	39.5	4.3	49.5	4.3	Small mask, closed
									30.5	4.8	35.3	4.8	42.8	4.8	43.0	4.8	Small mask, closed
							23.1	5.0	28.2	5.3	31.7	5.4	36.7	5.2	39.7	5.4	Large mask, closed
D. W. L.	F	160	5′6″	31			24.6	5.7	28.5	5.7	33.8	4.9	32.0	4.8	43.3	4.7	Small mask, closed
							24.8	5.5	30.2	5.3	39.2	4.9	40.7	5.1	46.2	5.4	Small mask, closed
A. S.	F	100	5′	29	16.2	4.5	27.3	3.6	37.1	4.0	49.3	4.3	54.4	4.0	61.6	3.9	Small mask, closed
									42.0	3.2	33.6	3.6	46.1	3.8	64.5	4.0	Small mask, closed
									30.5	3.3							
A. T.	M	165	5′10″	23			24.9	5.9	31.6	5.9	34.7	5.7	39.2	5.7	42.7	5.0	Small mask, closed
									35.3	5.3	42.0	5.2	44.1	5.2	46.0	4.9	Small mask, closed

Fig. 3.

METHOD OF INVESTIGATION

To test the efficiency of the apparatus, samples of expired air were taken from the test subject after five minutes of oxygen therapy at a fixed rate of oxygen flow. The inhaler was removed at the end of a normal inhalation, and the last 150 to 200 c.c. of air forcibly expired collected for analysis. The Orsat apparatus for gas analysis, as described in the United States Bureau of Mines Technical Paper No. 320, was used to determine the oxygen and carbon dioxide content of the sample. At least two series of samples were collected from each test subject. The results are given in Table I, but are better visualized in Fig. 4. The graph was obtained by plotting the averaged results of each test subject at the various rates of oxygen administration; however, only the values found while using the small mask (closed) were taken for the graph.

COMMENT

In viewing Table I and Fig. 4, it is seen that the alveolar oxygen content at 3 liters per minute is twice normal, and at 7 liters per minute, it is three times normal. As was expected, the higher concentrations were obtained in

the smaller subjects. That alveolar air was obtained for testing is shown by the concentrations of carbon dioxide in the samples. The minor variations in the alveolar carbon dioxide were such as would be expected with untrained subjects at different times during the day. With normal subjects oxygen flows at 4.5 to 7 liters per minute produced alveolar concentrations of 34 to 44 per cent oxygen, which concentrations are equivalent to those found with tent atmospheres of 40 to 50 per cent oxygen. The average pneumonia patient on the ward has been found to require 4 to 8 liters of oxygen per minute by this method to satisfy the therapeutic benefits expected from oxygen therapy.

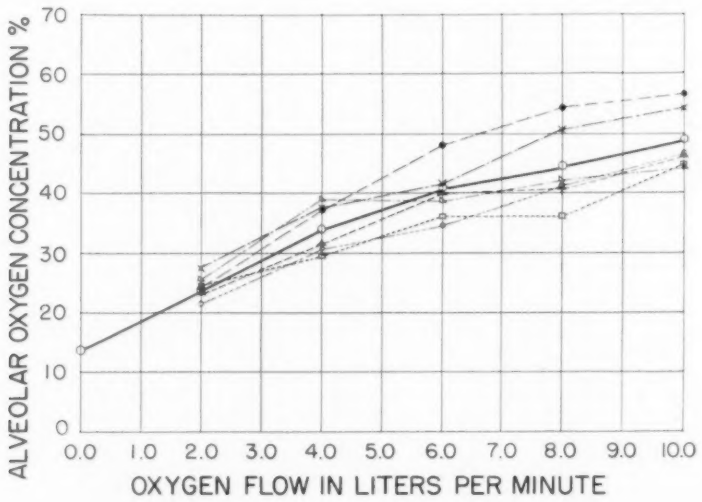

Fig. 4.

Many factors in addition to the oxygen tension tend to make oxygen therapy effective. Some of the more important basic requirements are overlooked in many types of equipment. The inhaler described satisfies many of these requirements. It leaves the patient's body accessible and in the room atmosphere. The split mask allows the patient, simply by raising the front section of the mask, to be given food, water, medication, and care without disturbing, to any marked degree, the concentration of oxygen in the respired air. The complete inhaler is small enough to cause little interference with the freedom of the patient. The use of the inhaler causes no irritation of the mucous membranes if the oxygen is partially humidified. Drying of the mucous membranes is noticed when no moisture is added to the dry gas. The inhaler does not excite patients, has no large reservoir of oxygen to build up before effective therapeutic concentrations are reached, requires little attention or experienced operators, and functions as well on mouth breathers as on nose breathers.

Excessive skin secretions necessitate cleaning and drying of the forehead before attaching the adhesive tape, or the use of one of the other methods of attachment. Odd-shaped faces when encountered require simple adjustment of the inhaler, such as the moving of the head plate, slight bending of the stem, or substitution of a larger mask. Some criticism has arisen due to the

loose contact between the patient's face and the mask. This looseness, though it must not be extreme, is a necessary part of the system and plays an important role in eliminating a closed system and the rebreathing of metabolites.

The economy of the inhaler in oxygen use, low original cost, and elimination of especially trained operators, together with its effectiveness in oxygen therapy, has made it possible to adequately supply the needs of many patients where formerly only a few of these patients could be given this care.

SUMMARY

1. A new apparatus for oxygen therapy is described.

2. Therapeutic concentrations of oxygen are obtained on comparatively low oxygen flows.

3. The apparatus is convenient, inexpensive, and comfortable.

We wish to express our appreciation to Dr. C. H. Thienes and Dr. D. R. Drury, of the University of Southern California School of Medicine, for their advice and guidance.

EFFECTS OF SMOKING ON BLOOD SUGAR AND PERIPHERAL TEMPERATURES OF CONVALESCENTS*

H. B. Cates, M.D., and J. G. Giovanaszzi, M.D., Los Angeles, Calif.

INITIAL interest was centered upon whether there is a correlation between changes in skin temperatures and rise in blood sugar induced by cigarette smoking. As the study progressed, however, the observations made upon the convalescent proved the more important. Their negligible response to smoking prompted us to test several volunteer medical students, and these had vascular responses to smoking, similar to the reactions observed by other workers.

The capillary blood sugar level of fasting nondiabetic patients has been determined by Nielsen[1] to be quite uniform and without oscillations. On a uniform diet the degree of variance of fasting blood sugar values was not over 10 mg. per 100 c.c. Working with fasting adults and recording respiratory quotients and blood sugar levels, Haggard and Greenberg[2] reported respiratory quotients between 0.78 and 0.82. The blood sugar values ranged from 0.08 to 0.10 per cent. Under identical conditions after smoking there was a rise of the blood sugar above 0.13 per cent. This rise is accredited to an increased adrenalin output possibly due to nicotine stimulation. However, Dill, Edwards, and Forbes[3] made observations upon the capillary blood sugar level in 10 subjects after smoking one cigarette, and found that nine-tenths of the subjects were within 5 per cent of the control level. There was no significant trend. In addition, lactic acid values were not appreciably altered by smoking. They conclude that carbohydrate metabolism is not changed by cigarette smoking.

*From the Department of Medicine of the University of Southern California.
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TABLE I

SUBJECT	AGE	MAX. TEMP. CHANGE OF FINGER	MAX. TEMP. CHANGE OF TOE	PULSE CHANGE	SYSTOLIC CHANGE	DIASTOLIC CHANGE	AVERAGE BLOOD PRESSURE	BLOOD SUGARS				
								CONTROL	15″	30″	45″	
Controls												
1	28	−4.6° C.	−1.9° C.	+32	+26	+14	108/68	97	99	101	100	Healthy control
2	28	−4.2° C.	−2.3° C.	+24	+35	+25	120/75	94	97	90	89	Healthy control
3	26	−6.5° C.	−4.0° C.	+22	+14	+10	116/64	100	125	95	125	Healthy control
4	27	−1.8° C.	−3.5° C.	+21	+ 6	+ 4	122/76	95	93	96	96	Healthy control
5	35	−5.1° C.	−0.5° C.	+12	+13	+ 4	107/78	98	99	100		Healthy control
6	26	−2.5° C.	−2.5° C.	+16	+22	+14	112/58	97	88	85		
Hypertensives												
7	28	+0.3° C.	−0.2° C.	0	− 5	0	225/115	86	88		91	Hypertensive and cardiac decompensation
			−1.0° C.	+ 4	0	0	220/130	95	97		97	Hypertensive heart disease
8	79	+0.1° C.	−0.1° C.	0	0	0	165/88					
		+0.9° C.	+0.4° C.		+ 3	+ 2	155/80					
9	70		+1.5° C.	0	+ 5	− 5	170/100	77	143		143	Hypertensive coronary heart disease
10	65	0	0				190/120					Hypertensive heart disease, fibrillation
11	69	−0.3° C.	+0.3° C.	0	+15	+12	175/110					Hypertensive heart disease
12	53	0	0	0	+15	0	175/100					Hypertensive heart disease
			0	0	+10	+ 5	190/45					Cardiac decompensation, fibrillation
13	67	0	+0.3° C.	− 2	+ 8	0	180/100					Hypertensive coronary heart disease
14	44		0				238/140	104	108		400*	Hypertensive heart disease
			−3.5° C.		0	+10	250/150					
15	28	0	0	0	0	− 5	210/115	102	90		444*	Hypertensive heart disease
16	32		−3.5° C.		+10	+ 5	170/95	100	105		100	Hypertensive heart disease
			−3 ° C.				170/95					
17	70	−1.4° C.	−0.9° C.	+10	0		165/125					Hypertensive heart disease; bundle branch block, fibrillation

*After the injection of 50 c.c. of 50 per cent glucose.

TABLE I—CONT'D

							Nonhypertensives					
18	57	+0.2° C.	+0.1° C.									
19	57	0	−0.5° C.	+ 8	+ 6	+ 2	138/83					Duodenal ulcer
20	32	−0.4° C.	−0.4° C.	+ 8	+12	+ 5	130/80					Observation
21	79		−2.5° C.				116/42					Undetermined
22	81	0	+1.5° C.		−10	−10	145/80					Pulmonary embolism
23	75	−0.5° C.	0				135/80					Senile Parkinson scoliosis
24	53		−0.5° C.				130/100	98	97		97	Coronary heart disease, fibrillation
			−0.1° C.				130/100	103	101		103	Chronic alcoholism, pellagra, improved
25	39		+0.7° C.		− 2	−10	92/0	100	102		400*	Cardiac decompensation, coronary disease
							88/76	105	102		400*	
26	38	0	−0.5° C.	− 1			100/60					Rheumatic heart disease, mitral stenosis, fibrillation
27	25		−4.7° C.	+ 6	0	0	110/75	102	97			Psychoneurosis
			−1.0° C.				110/75	60	71		85	
28	35	−4.6° C.	−3.5° C.								71	Bleeding peptic ulcer
29	28		−2.7° C.	− 2	− 9	− 5	120/80					Rheumatic fever
30	60		−1.5° C.	−10	0	−10	120/80	100	110		400*	Carcinoma of penis
31	49		−0.5° C.				130/80	71	74		74	Effort syndrome
			−1.0° C.				140/80	98	117		111	
32	22		−0.5° C.				90/60	101	104		105	Moderate advanced tuberculosis, duodenal ulcer
33	31	−0.5° C.	−1.0° C.				120/70					Attempted suicide, depressed psychosis
		0	−0.5° C.	0	+ 4	0	100/60	100	90		95	
34	49	+1.0° C.	+1.0° C.				100/70	95	83		95	Intestinal adhesions, psychoneurosis
		−2.0° C.	−0.5° C.	− 8			100/60	90	80		93	
35	39	0	−0.4° C.	+ 8	0	0	100/70	97	111		102	Psychoneurosis

Blood sugars of 400 mg. are subsequent to the injection of glucose intravenously.

There is an apparent agreement of reports on the lowering of peripheral temperature of subjects after smoking. Maddock and Coller[4] find a fall of 5.9° C. of the fingers and 0.50° C. of the toes while smoking.

Barker,[5] testing a group of 20 patients, found a significant drop of 1.8° C. after smoking three cigarettes in rapid succession. Five patients had temperature changes in both fingers and toes. Johnson and Short,[6] when comparing smokers with nonsmokers, reported that 88 per cent of smokers showed an average drop of 2.5° C., while with nonsmokers only 34 per cent showed a drop, the average being 1.8° C. This is attributed to the fact that the latter did not inhale. Wright and Moffat[7] conclude that practically all subjects who inhale will show a temperature drop, though not necessarily every time the test is taken. An individual may have a lowering in skin temperature of 5.5° to 6.6° C. upon one day and as little as 0.6° to 1.7° C. with the same tobacco product upon the next day. Apparently in 16 out of 50 tests the subjects complained of mild toxic symptoms. This was manifest by tingling of the fingers and slight vertigo. In 7 instances nausea, vomiting, cold sweats, and syncope occurred. In another report, Maddock, Malcolm, and Coller[8] state that cigarette smoking produces equivalent decrease in skin temperature of women and men. They find more marked changes in Jewish subjects than in gentiles. They also note increase in blood pressure and pulse rate in all of the subjects tested. Their protocols show that 28 out of 29 patients were in the third decade of life and that 15 cases out of 29 had increases of systolic pressure which were less than 15 mg. mercury. Eight of the 29 had temperature reduction of either fingers or toes which was less than 1.5° C. Herrell[9] reports an idiosyncrasy to tobacco in a man 57 years of age, whose chief complaint was periods of rather severe dizziness. After smoking a cigarette the blood pressure was found to rise from 118/70 to 180/110, while the peripheral temperature, tips of fingers, decreased on an average of 1° to 1.5° C. between the first and second readings. Herrell and Cusick[10] reported 3 more sensitive subjects who reacted to smoking with elevation of blood pressures and associated spasm of the retinal vessels. This response is regarded as one of idiosyncrasy to tobacco.

PROCEDURE

All of the subjects studied, with exception of 6 students, were patients selected from a medical ward of the Los Angeles General Hospital, with the requirement that they were afebrile and received no medication except digitalis. The majority of these had either hypertension or heart disease, for with these, it was believed temperature and blood sugar fluctuations might prove more marked than in other diseases. It must be emphasized that no patient was urged to smoke more than was his custom, and similarly no untoward effect was reported by the subject.

The age incidence of the subjects varied from the third to the seventh decade. All were exposed for an hour or more to a constant temperature. Tests were made at a fixed temperature between the extremes of 70° and 80° F. The room temperature oscillations in the majority of instances were within one degree. Fasting capillary blood sugars were determined by the colorimetric micromethod of Benedict. A control specimen, the second fifteen

minutes, and a third, thirty minutes later, were taken. A Taylor's dermotherm was used, and the temperature of the tips of the first three fingers and toes was read for changes in conjunction with the blood sugar tests. Upon others, records were made of the temperature changes of the fingers and toes of the right side. In addition, the pulse rate and blood pressures were determined. The pressures were taken with a mercury sphygmomanometer upon the left arm. The apical heart rate was counted for a period of thirty seconds. Temperature determinations were made at five-minute intervals, allowing at least twenty minutes to establish a control of the peripheral temperature before requesting the subject to begin smoking. Cigarettes of the popular brands were offered, and the subject consumed from two to five without interruption. About two-thirds of each cigarette was consumed.

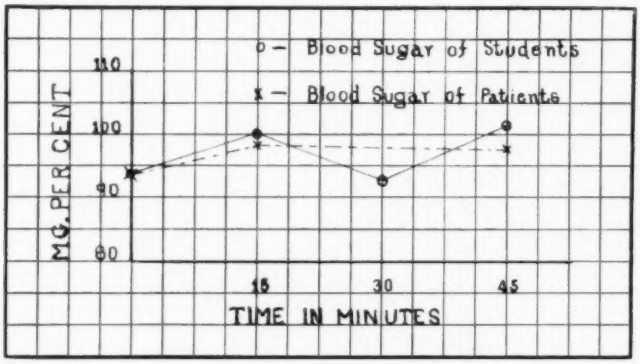

Fig. 1.—Relationship between the capillary blood sugar levels before and during cigarette smoking.

RESULTS

The lowering of peripheral temperature during smoking tests made upon 6 medical students was pronounced. The least decrease was 1.8° C., and the maximum, 6.5° C. Fluctuations of skin temperature under standard conditions which were less than 1.5° C. were not regarded as significant and were not ascribed to tobacco.[11] The pulse rate counted at five-minute intervals was increased by smoking, with a maximal increase of 32 and a minimum of 12 beats per minute. Blood pressure was elevated in most of the young subjects. The second subject had a systolic elevation of 35 and a diastolic increase of 25. The least change, noted in the fifth subject, was 13 systolic and 4 diastolic. These findings compare well with many previous reports, but serve to contrast the paucity of fluctuations noted upon the convalescent hospital patients.

The hypertensive group of patients, while smoking, showed no greater variation of skin temperature than occurred during the preceding control period of forty-five minutes. In this group, highest blood pressure rise was 15 mm., the average 8 mm. This is to be contrasted with figures in the student group when the highest was 35 mm. and the average rise was 19 mm.

The third group, those patients having normal blood pressures, and the majority of whom were admitted to the hospital suffering cardiac decompensation, gave a negative temperature response. Of 24 studies only 4 had a

temperature decrease of their toes over 1.5°C. Two of 12 had depressed finger temperatures.

A composite curve representing 26 curves of capillary blood sugar determinations on 20 subjects, establishes no elevation of the fasting dextrose values after smoking two to five cigarettes. Artificial elevation of the blood sugars to 400 mg. per cent by intravenous injection of 50 c.c. of 50 per cent glucose likewise had no indirect effect upon peripheral temperature.

DISCUSSION

At present clinicians have no unanimity of opinion as to whether smoking is detrimental to patients suffering from certain diseases. Abstinence from tobacco is considered by some physicians to be chiefly responsible for the alleviation of symptoms in Buerger's disease, hypertensive heart disease, and peptic ulcer. The conclusion of Thienes and Butt,[12] after making well-controlled animal experiments, is that no demonstrable circulation lesions may be attributed to chronic nicotine poisoning in the animals tested. On the basis of our observations we conclude that smoking affects the peripheral vascular system less in cardiac convalescents than in young healthy males who have not been confined to bed. Again, the older the subject is, the less susceptible his vascular tone. Results obtained from the subjects of the second and third decade may not by inference be applied to later decades of life.

CONCLUSION

1. Convalescents show less response to tobacco than young healthy adults.

2. Smoking does not elevate capillary blood sugars, and there is no relationship evident between blood sugar levels and peripheral temperatures.

3. Patients having hypertension show no more vascular response to smoking than the nonhypertensive.

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LABORATORY METHODS

A MANUAL OF NEUROHISTOLOGIC TECHNIQUE*

OSCAR A. TURNER, M.D., NEW HAVEN, CONN.

Contents.—I. General Considerations. II. Stains for Nerve Cells. III. Stains for Myelin Sheaths. IV. Neurofibril Stains. V. Staining of Fat. VI. Nonmetallic Glia Stains. VII. Metallic Glia Stains. VIII. Connective Tissue Stains. IX. Pituitary and Pineal Stains. X. Spirochete Stains. XI. Miscellaneous Staining Methods. XII. Formulas. Appendix. References.

CHAPTER I

GENERAL CONSIDERATIONS

Fixation of Tissues.—The importance of the immediate fixation of tissues after death cannot be overemphasized. Tissue which has been allowed to remain unfixed for some hours often gives indifferent and unsatisfactory results and is especially prone to artifact formation. This is particularly true in respect to the silver impregnation methods.

Although the methods selected here were primarily for use in a general pathologic laboratory where formalin is the routine fixative used, reference will be made to certain special methods of fixation. Next to formalin, Zenker's fluid is probably the most commonly employed tissue fixative and is particularly useful where many of the connective tissue and nonmetallic glial stains are to be used. Formalin-ammonium bromide is necessary as a fixative for most of the original metallic glial methods, although at the present time there are several modifications of these stains which give excellent results on formalin-fixed material. Fixation in Regaud's solution or Zenker's solution is required for many of the more selective pituitary stains, although there are several satisfactory methods which may be employed on formalin-fixed material. The present methods of treating tissue prior to staining makes possible the use of tissues fixed in formalin for the demonstration of practically every element of the nervous system.

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Dr. Harry Goldblatt, Dr. Howard T. Karsner, and Dr. Albert Steegmann, of the Institute of Pathology, and the late Dr. T. Wingate Todd, of the Anatomical Laboratories, gave freely of their time and experience and proved to be a source of invaluable assistance and advice in the preparation of this manuscript. For their helpful criticism and interest the author owes a debt of gratitude. The various members of the technical departments at the Institute of Pathology, particularly Miss Irene Boten, Mr. Donald Burt, and Miss Emily Squire, were patient in my demands upon their time and experience. Dr. Harry Zimmerman, of the Department of Pathology, Yale University, was kind enough to review the methods and offer valuable advice. Finally, thanks are due to Miss Edith Orling and Miss Frances Turner, who did much of the technical work in the preparation of the manuscript. With all this assistance, any errors that have crept into the text can only be mine.—*Oscar A. Turner, M.D.*

This article will appear in several installments. A list of selected references will appear in the last installment.

Removal of Tissue.—The method of removing blocks of tissue depends upon the nature and location of the lesion to be studied. For routine study of the brain, Spielmeyer recommends taking blocks of tissue from:

1. Posterior part of the first frontal convolution.
2. Both central convolutions, approximately at the borderline of the upper and middle thirds.
3. First temporal convolution.
4. Region of cornu ammonis.
5. Region of the calcarine tissue.
6. Middle of the basal ganglia together with the island of Reil.
7. Posterior portion of the basal ganglia with the island of Reil.
8. Region of the oculomotor nuclei.
9. Inferior olives.
10. Vermis and cerebellar hemispheres.
11. Spinal cord segments: cervical and lumbar enlargements, upper cervical, upper and lower dorsal and sacral regions. Additional blocks will depend upon the nature of the individual specimen to be studied. For the study of tumors, blocks should always include a considerable portion of the adjacent nervous tissue and at least half the diameter of the tumor if this is feasible. Several blocks of tissue should be taken from various portions of the larger tumors, and a complete cross section is recommended for the smaller growths. Longitudinal, as well as cross sections of peripheral nerves, are often found useful. The blocks of tissue should not exceed 5 mm. in thickness and should be fixed in at least ten times their volume of fixative. The size of the tissue block, other than thickness, depends upon the individual lesion to be studied, the ability of the technician to handle very large or very small sections, and the facilities at hand for the preparation of very large sections.

Laboratory Equipment.—Special equipment, other than that found in the average laboratory for histologic technique, is not necessary for the staining of the nervous system. Small glass dishes, holding 30 to 40 c.c., have been found useful for frozen section work. Where larger receptacles are needed, small beakers or large Petri dishes may be used. Glass rods bent to form a hook are used for handling frozen sections, and a small camel's-hair brush is employed to draw these on the glass slide. Bibulous paper is best for blotting sections, although filter paper may be used.

Gelatin Embedding.—For many methods in which paraffin embedding is contraindicated or not feasible, it is necessary to embed the tissue in gelatin before making frozen sections. The following method has been found to give excellent results. Blocks of formalin-fixed tissue are washed in running water for twenty-four hours, after which they are placed in thin gelatin solution (12 per cent) for a similar length of time in the 37° C. incubator. They are then transferred to thick gelatin solution (25 per cent) and allowed

to remain twenty-four hours at the same temperature. The blocks are then placed in small dishes and allowed to harden in the thick gelatin for one to two hours in the icebox. The excess gelatin is then trimmed off, and the blocks are further hardened in 10 per cent formalin for one to two days and washed for one to two hours in running water before sectioning. The blocks are sectioned on the freezing microtome in the usual manner. The method is used in making frozen sections of small fragments of tissue or where the tissue sections crumple and tear when prepared by the usual frozen section method.

The preparation of the gelatin solution is quite simple. A weighed amount of table gelatin is added to three times its amount of a 1 per cent aqueous solution of carbolic acid. This is allowed to remain in the incubator at 37° C. until dissolved to form a thick fluid mass. To make the thin gelatin (12 per cent), dilute the 25 per cent solution with an equal part of the 1 per cent aqueous carbolic acid.

Chapter II

Stains for Nerve Cells

The most widely used methods for the demonstration of nerve cells and cortical architectonics are those based upon the principle devised by Nissl. Excellent results can be obtained upon formalin-fixed tissue, either embedded in celloidin or cut with the freezing microtome. The following modifications of the original method have been found to give consistent results with the minimum of effort. With a little practice differentiation can be carried to the point where the cell bodies and the Nissl substance stand out in sharp contrast to a colorless, or nearly colorless, background.

Cresyl Violet Stain for Frozen Sections.—Blocks of tissue, not over 5 mm. thick, are washed in running water for a half hour, after which they are allowed to remain in distilled water for about one hour. Frozen sections are cut at 15 microns.

1. Place sections in 50 per cent alcohol and allow to remain in the incubator at 37° C. for two days.

2. Transfer the warm sections to a 3:5000 aqueous solution of cresyl violet. (Filter stain through a single layer of very coarse filter paper before using.)

3. Heat until steam arises from the stain. Do not boil. Allow to cool.

4. Rinse sections in distilled water.

5. Transfer quickly to 70 per cent alcohol. Shake sections for about one minute.

6. Differentiate the sections in 96 per cent alcohol. This is the most important step and a little practice will enable one to determine how far the decolorization should be carried.

7. Pass sections through absolute alcohol for a few seconds to complete dehydration.

8. Clear in xylol. The sections should be held until the xylol has penetrated, so as to prevent agitation which may tear fragile sections.

9. Draw sections on a clean glass slide, blot with bibulous paper, cover with a drop or two of xylol, and mount in balsam. Cedarwood oil is better than balsam for mounting Nissl stains, but has the disadvantage of drying very slowly and imperfectly. Small weights should be placed on the cover slips to keep the sections flat while drying.

Thionine Stain for Celloidin Sections.—Sections of the celloidin-embedded tissue, cut at 15 microns, are placed in 70 per cent alcohol and kept in the incubator at 37° C. for twenty-four to forty-eight hours.

1. Transfer warm sections quickly to a 1:5000 aqueous solution of thionine.

2. Heat until steam arises, never allowing the stain to come to a boil. Allow to stand until cool.

3. Transfer to distilled water.

4. Quickly transfer sections to 70 per cent methyl (wood) alcohol and agitate for a few seconds.

5. Differentiate in 96 per cent methyl alcohol. (Methyl alcohol differentiates rapidly and is better suited for formalin-fixed tissue. Ethyl alcohol is less rapid and is used to differentiate alcohol-fixed material.)

6. Complete dehydration for a few seconds in absolute alcohol.

7. Clear in xylol.

8. Draw sections on a slide and after straightening, trim off the excess celloidin, blot quickly, and cover with balsam. Warm very gently and place cover slip over the section. Allow to stand about twenty minutes, heat gently again, and after pressing out the air bubbles, keep overnight under weights.

Toluidine Blue Stain for Alcohol-Fixed Tissue.—Although excellent results may be obtained from formalin-fixed tissue, stained either with cresyl violet or thionine, by far the best results are obtained through the use of tissue which has been fixed in 96 per cent alcohol and stained with toluidine blue.

Blocks of tissue not over 5 mm. thick are fixed for several days in at least ten times their volume of 96 per cent alcohol. The alcohol should be changed frequently. Following fixation, the tissue is embedded in celloidin and stained according to the method given above for celloidin-embedded tissue. However, a 1:1000 aqueous solution of toluidine blue is used for staining, and ethyl (grain) alcohol is used for differentiation instead of methyl (wood) alcohol. A 1:5000 aqueous solution of thionine may also be employed as a stain.

A more rapid, although less satisfactory, method may also be employed where a sliding microtome is available. After fixation the tissue is dried care-

fully between two sheets of filter paper and placed in its proper position on the wooden block. It is then covered with thin (12 per cent) celloidin and allowed to dry. The tissue is then sectioned on the sliding microtome at 20 microns, without further embedding. The sections are stained as for celloidin-embedded tissue. The tissue may also be fixed to the wooden block with gum arabic and hardened in 96 per cent alcohol for a few minutes.

The following remarks apply to Nissl stains in general. If the differentiation is carried to the right degree, the background should be colorless or nearly so. The ganglion cells stand out clearly and are in sharp contrast to the background. If the sections are passed through chloroform after dehydration in the absolute alcohol, the blue color of the cells assumes a lilac cast, which gives considerable contrast. The Nissl substance takes a deep stain, and nuclear detail is clear. In the white matter the nuclei of the various glial cells take the stain deeply, but only occasionally can the suggestion of cell processes be made out. The walls of the larger vessels take the stain rather intensely, while in the cortex the capillaries can be identified with ease. Erythrocytes are stained deep blue, but disintegrated blood often takes a greenish-blue color. Pathologic pigments of various sorts usually take an intense blue, but not always so. Cellular lipoid inclusions are not stained.

The stained sections have a tendency to fade and should be stored in the dark and never placed in direct sunlight. This is especially true of cresyl violet, which is very sensitive to sunlight. Sections stained as described above and kept in the dark have retained their color quite well for a number of years. It is best not to place the stained sections in an incubator.

Staining should be carried out in a large evaporating dish, and differentiation in glass containers over a white background. Coloration of the celloidin in embedded material may be avoided by the use of fresh alcohols, especially the 95 per cent and the absolute. A 1 per cent aqueous stock solution of the dyes keeps for long periods and is easily diluted to the desired strength for staining.

Chapter III

Stains for Myelin Sheaths

Myelin sheath stains find their greatest use in general neuropathology, although their use in general pathology is by no means limited. The staining methods described below vary widely in the time required for staining, and all are easily carried out. With experience, there is little trouble in obtaining consistently reliable results.

Pal-Weigert Method.—This method is applicable to either frozen or celloidin sections. The tissue should be thoroughly fixed in 10 per cent formalin.

1. Blocks of tissue, not over 1 cm. thick, are placed in Weigert's primary mordant and allowed to remain for four to five days at room temperature. Weigert's primary mordant is prepared as follows:

Potassium bichromate	5 gm.
Chromium fluoride (fluorchrome)	2 gm.
Water, to make	100 c.c.

2. The tissue is then transferred to Weigert's secondary mordant, where it is allowed to remain for twenty-four to forty-eight hours:

Copper acetate	5.0 gm.
Glacial acetic acid 36%	5.0 c.c.
Chromium fluoride	2.5 gm.
Water, to make	100.0 c.c.

Dissolve the chromium fluoride in boiling water, add the glacial acetic acid and copper acetate.

3. The tissue is dehydrated in alcohols of ascending strengths and embedded in celloidin in the usual manner. However, frozen sections may be cut at 30 or more microns and carried through the following procedures.

4. The sections are transferred to Weigert's hematoxylin and allowed to remain twenty-four to forty-eight hours. The hematoxylin is prepared as follows:

10% hematoxylin in absolute alcohol, ripened	10 c.c.
Water	90 c.c.

5. Wash until uniformly blue in water made alkaline by the addition of from 1 to 3 per cent of a saturated solution of lithium carbonate.

6. Differentiate in 0.25 per cent potassium permanganate until the gray matter is brownish yellow.

7. Transfer to the following solution:

Oxalic acid	1 gm.
Potassium sulfite	1 gm.
Water	200 c.c.

Allow to remain until the gray matter is colorless, or nearly so.

8. Wash in water and blue again in lithium carbonate solution.

9. Wash in water, dehydrate in alcohol, and clear in xylol. Mount in neutral balsam.

The myelinated nerve fibers appear dark blue to bluish black upon a colorless or slightly yellow background. The differentiation is sharp, and the finest nerve fibers are deeply stained. Cells and axis cylinders do not take the stain. Areas of demyelinization appear pale yellow and devoid of stain, although where the process is incomplete, scattered nerve fibers may be seen.

In differentiating the sections, it is best not to allow them to remain in the potassium permanganate longer than one minute before washing and placing them in the oxalic acid solution. They should then be washed, blued in lithium carbonate solution, and again treated with the permanganate. This is to prevent the sections from assuming a yellowish-brown color, which will

resist decolorization by the oxalic acid. It is usually necessary to repeat this process several times before the correct degree of differentiation is obtained. The degree of decolorization can be carried out to suit the individual taste. The main disadvantages with this method lie in the length of time required for its completion and the fact that the tissue cannot be used for many other stains once it has passed through the Weigert mordants. The method has withstood the test of time, and is one of the most reliable.

The mordants retain their strength for considerable periods of time as does the oxalic acid solution. The potassium permanganate solution should be prepared as a 2 per cent stock solution and diluted just before use. The hematoxylin should be prepared from the alcoholic solution and also diluted just before use. It should not be used more than once.

Spielmeyer's Method.—The method gives good results and can be completed in two days. Formalin-fixed tissue cut with the freezing microtome is used. The tissue should be washed in running water for at least thirty minutes before sectioning to remove the formalin. Sections should be cut at 30 or more microns.

1. Allow frozen sections to remain in tap water for two hours before staining.

2. Transfer to 2.5 per cent iron alum solution (ferric potassium sulfate) and allow to remain overnight. Care should be taken to see that the sections lie flat in this solution as there is a tendency for them to harden and wrinkles may be difficult to remove.

3. Rinse in distilled water and transfer to 70 per cent alcohol where they should remain ten minutes. Agitate during this time.

4. Transfer to an old hematoxylin solution made up as follows:

10% solution of hematoxylin in absolute alcohol	5 c.c.
Distilled water	100 c.c.

Spielmeyer states that if the hematoxylin solution is old and stains well, one to two hours is sufficient. It may be necessary to stain as long as six to ten hours. The sections should be uniformly black when removed from the stain. Inasmuch as there is a tendency for the sections to become extremely brittle in the hematoxylin, staining should not be prolonged more than is necessary.

5. Rinse in distilled water and differentiate in iron alum solution. Here it is well to use a 4 per cent solution for the early stages and a weaker solution, 2.5 per cent or less, toward the end. Sections should be handled carefully to prevent them from fragmenting. They may be transferred from one solution to another on slips of blotting paper.

6. Wash sections thoroughly in three changes of distilled water.

7. Dehydrate in ascending alcohols and clear in xylol. Mount in neutral balsam.

The results are similar to those of the Pal-Weigert method. Occasionally there is a slightly deeper yellow color to the background, but often this can be prevented by liberal washings where noted above. The hematoxylin solution may be used repeatedly if filtered before returning to the stock bottle, although some authors recommend using fresh hematoxylin solution each time. Its staining power increases with age. Washing the sections in 70 per cent alcohol removes fatty substances, and incomplete treatment at this stage results in uneven staining. If this occurs, the sections should be carried back through water to 70 per cent alcohol and again stained and differentiated. During differentiation the sections should be moved about, and for larger and deeply stained sections it may be necessary to replace the differentiating fluid with fresh solution. The disadvantage in this method lies in the extreme brittleness of the sections after removal from the stain, making it difficult to obtain intact preparations from large specimens.

Weil's Rapid Method.—This method may be used on material embedded in celloidin or paraffin or cut on the freezing microtome. Paraffin sections give less satisfactory results, but if used, should be cut at about 15 microns. Celloidin-embedded material gives the best results. When frozen sections are used, they should be carried through 95 per cent alcohol, absolute alcohol, xylol, and back down again through the descending concentrations of alcohol to water. The method is as follows:

1. Wash sections in distilled water.

2. Stain for ten to thirty minutes at 50° to 55° C. in a mixture of equal parts of (a) and (b). The stain should not be filtered and should not be used more than once. The individual components keep indefinitely.

   (a) Ferric ammonium sulfate solution, 4%.

   (b) 1% solution of hematoxylin which has been made by adding 90 c.c. of distilled water to 10 c.c. of a ripened 10% solution of hematoxylin in absolute alcohol.

3. Wash twice in tap water.

4. Differentiate in a 4 per cent aqueous solution of iron alum. For celloidin material, differentiate until the gray matter or areas of demyelinization can be just distinguished; for paraffin sections, long enough to remove the stain from the back of the slide. Care should be taken not to overdifferentiate in iron alum, for in doing so the fine fibers lose their stain.

5. Wash in three changes of tap water.

6. Complete the differentiation in the following solution:

Borax	10.0 gm.
Potassium ferricyanide	12.5 gm.
Distilled water	1000.0 c.c.

This solution keep indefinitely.

The decolorization should be controlled under the microscope.

7. Wash in two changes of tap water.

8. Wash for thirty seconds in dilute ammonia water. (Add 6 drops of 28 per cent ammonia to 100 c.c. of water.)

9. Wash in distilled water.

10. Dehydrate in alcohol, clear in xylol, and mount in neutral Canada balsam.

The above method has the advantage of being rapid and of giving fairly consistent results. The myelinated fibers are stained a deep blue, and if the section has not been overdifferentiated in iron alum solution, the fine myelinated fibers can be traced with ease. The background is usually a light yellow color, and if care is taken to wash the tissues thoroughly after each stage of differentiation, the color of the background is not too deep and the contrast is excellent. It is important that the hematoxylin used should be completely ripened, at least for six months. Failure to obtain staining of the tissue in the hematoxylin is usually due to the use of insufficiently ripened solution. The stain should not be filtered before use and it cannot be used more than once. For 10 celloidin sections cut at about 30 microns, 50 to 75 c.c. of stain should be used. The length of time required for the sections to take the stain varies somewhat with the method used in sectioning the tissue. Celloidin-embedded material should be left in the stain from twenty to thirty minutes; paraffin sections require ten to fifteen minutes. Large sections may become somewhat brittle, but never enough to cause serious trouble if the material is handled carefully. Large sections can be transferred from solution to solution on slips of blotting paper. It is not advisable to use paraffin sections if other tissue is available.

Wolter-Kulschitzky Stain for Myelin Sheaths.—This is the best of the various modifications of the original Weigert method for staining myelin sheaths. The method utilizes formalin-fixed tissue which is embedded in celloidin. It has the disadvantage that, like the Weigert method, the tissue blocks must be mordanted before embedding, thus making them useless for other stains.

1. Place blocks of formalin-fixed tissue directly into Weigert's primary mordant. They are left here in the dark for ten to fourteen days, depending upon the size of the block.

2. Wash in running water for one-half to two hours.

3. Place in 70 per cent alcohol for one day.

4. Leave in 96 per cent alcohol for three to six days. Change the alcohol after the first day.

5. Leave in absolute alcohol for two days. Change alcohol after the first day.

6. Transfer tissue to absolute alcohol ether (1:1) and allow to remain for one to two days, the latter for the larger blocks of tissue.

7. Place in thin celloidin and allow to remain fourteen days or longer.

8. Transfer to thick celloidin for eight to fourteen days. The tissue must be kept in the dark up to the time the infiltration with celloidin is completed.

9. Embed in celloidin and section in the usual manner. Allow the blocks to harden for one day in 70 per cent alcohol before sectioning. The sections may be cut at from 25 to 100 microns, depending upon the purpose of the stain. Those cut at 25 to 30 microns may be used for detailed microscopic study, while those cut at from 50 to 100 microns are for low magnification or naked-eye examination.

10. The sections are received in 70 per cent alcohol. Transfer quickly through distilled water to Müller's fluid where they remain for eight days or longer.

11. Wash in two changes of distilled water and pass briefly through 70 per cent alcohol. Transfer to Kulschitzky's hematoxylin and allow to remain overnight in the incubator at 37° C.

10% solution hematoxylin in absolute alcohol, ripened	10 c.c.
Glacial acetic acid	1 to 2 c.c.
Distilled water	100 c.c.

Heat together until dissolved.

12. Wash in two changes of distilled water.

13. Leave in Müller's fluid for two minutes.

14. Transfer to tap water and wash for five minutes or longer. Change the wash water until it remains clear.

15. Transfer to 0.25 per cent aqueous potassium permanganate solution for ten to twenty seconds.

16. Transfer rapidly through distilled water into a mixture of equal parts of 1 per cent oxalic acid and 1 per cent potassium sulfate. Allow to remain about five minutes. Change solution when it turns brown.

17. Wash in several changes of distilled water. Return sections to the permanganate solution, wash and again decolorize in the bleaching mixture. Repeat this process until the desired degree of differentiation is obtained. Wash the sections thoroughly in distilled water and allow to remain overnight.

18. Dehydrate in ascending concentrations of alcohol, pass through carboxylol, and clear in several changes of xylol. Mount in balsam.

The myelin sheaths are stained a dark blue against a practically colorless background. The fine myelinated fibers are deeply stained, and the contrast is excellent. Poorly infiltrated tissue is difficult to section and fragments easily. The sections tend to become brittle in the hematoxylin, and Wertham suggests transferring them to pieces of paper, upon which they may be stained and differentiated with less danger of fragmentation. The method can be used on very large blocks of tissue if care is taken that the infiltration with celloidin is complete.

As with other myelin sheath stains employing ripened hematoxylin, the absolute alcohol solution must be at least six months old. Older hematoxylin gives better results. The 0.25 per cent potassium permanganate solution should be prepared fresh from a 2 per cent stock solution and, like the bleaching mixture, should be changed frequently during the process of decolorization. The celloidin-embedded blocks of tissue may be stored in 70 per cent alcohol. As in all frozen section or celloidin work, small weights should be placed upon the cover slips after mounting until the balsam has hardened.

Marchi Method for Degenerating Myelin.—It is occasionally desirable, especially in tracing very small or scattered areas of degeneration, to use a method which gives a positive stain for the degenerated myelin. For this the Marchi method, utilizing osmic acid as a staining agent, is almost universally employed. Whereas in the previous methods the staining was limited to the sheaths of the uninjured myelinated nerve fibers, here the staining is confined to the myelin of the degenerated or partly degenerated fibers.

1. Small blocks of tissue, not more than 2 to 3 mm. thick, are fixed in Müller's fluid or 3 per cent potassium dichromate, changing the solution every three or four days. The time of fixation varies from one to two weeks.

2. The tissue is transferred directly without washing into the following solution:

Müller's fluid or 3 per cent potassium dichromate	2 parts
1 per cent osmic acid	1 part

Prepare fresh before use.

The tissue should be left in this solution for one to three weeks and the solution should be renewed weekly.

3. Wash stained blocks in running water for twenty-four hours.

4. Dehydrate as rapidly as possible and embed in celloidin.

5. Cut sections at from 10 to 5 microns, according to purpose, and mount in dammar or balsam.

The degenerating myelin is seen as small black dots and globules on a light yellow or yellowish-brown background composed of the unstained elements of the nervous system. The lipochrome granules of nerve cells may also be stained black or brown. The sections may be counterstained with safranine, neutral red, or van Gieson's stain as desired. The length of time of fixation should not be unduly prolonged since overfixation may produce artifacts in the form of small black granules which are difficult to differentiate at times from degenerating myelin. It also should be noted that fat cells in the connective tissue stroma of nerves also take the black stain. The length of time required in the osmic acid mixture varies with the size of the blocks of tissue and the types of tissue being treated. The spinal cord and peripheral nerves usually require from eight to fifteen days, while twenty-one to twenty-eight days is generally sufficient for the cerebellum and cerebrum.

Anderson suggests the addition of 10 to 20 c.c. of 1 per cent acetic acid to each 100 c.c. of Marchi fluid to facilitate penetration. Material which has been fixed in formalin for not more than two to three days may be used by transferring directly to the osmic acid mixture. The results here, however, are less reliable than with tissue which has been fixed in Müller's fluid or 3 per cent potassium bichromate.

It is not possible to give exact figures for the time the tissue remains in many of the reagents. Not only do the time relationships vary with the size of the tissue blocks, but also with the portion of the nervous system from which the tissue was removed. The type of mammal, age of the tissue, and maturity of the tissue play a part as well. For this reason, wherever indicated, the time limits are given. In general, the time requirements are not rigid and good results are not difficult to obtain.

Precautions should be taken in the interpretation of the results of this method. Aside from the black particles of degenerating myelin, there may be an irregular precipitate which may be difficult to differentiate from actual degeneration. Care must be exercised in handling the unfixed tissue to prevent displacement of the degenerating myelin. Although it is best to dehydrate and embed the stained tissue block as rapidly as possible, it is also important that the process of dehydration be complete. Small pieces of tissue may be embedded in paraffin if chloroform and not xylol is used as a clearing agent.

Aside from the time element, the chief disadvantage in this method lies in the ease with which artifacts are produced. However, in experienced hands it is fairly reliable. For routine pathologic work any of the myelin sheath stains are to be preferred, and for experimental work it is best to control the results with one of the myelin sheath stains.

Chapter IV

Neurofibril Stains

Staining methods for the demonstration of neurofibrils are important both to the study of general neuropathologic and tumor material.

Bielschowsky's Silver Method for Neurofibrils.—This method utilizes frozen sections cut at 10 microns from formalin-fixed tissue. The tissue should be washed in running water for one-half to one hour before sectioning, and the sections should remain in distilled water for two hours before staining. Paraffin-embedded material may be used also.

1. Transfer sections to 2 per cent silver nitrate solution and allow to remain for twenty-four to forty-eight hours. It is best to keep the sections in the dark while in this solution. They may be placed in 4 per cent silver nitrate in the incubator at 37° C. and left overnight to save time.

2. Rinse quickly in double distilled water.

3. Transfer sections to freshly prepared ammoniacal silver solution and allow to remain for ten to twenty minutes.

10% silver nitrate	5 c.c.
40% sodium hydroxide	5 drops

Wash the dark brown precipitate which is formed with three changes of double distilled water. Add water to make 5 c.c. and dissolve the precipitate by adding strong ammonium hydroxide drop by drop. Avoid an excess of ammonia. Dilute resultant solution to 20 c.c. with double distilled water.

The sections should be removed when they are deep brown in color.

4. Rinse rapidly in two changes of double distilled water, and reduce in 20 per cent formalin made up with tap water. Reduce for five to ten minutes. The sections should assume a slate gray or grayish-black color.

5. Wash in double distilled water for ten minutes.

6. Tone sections in the following solution:

1% gold chloride	15 drops
Distilled water	10 c.c.

The toning solution may be acidified with two or three drops of glacial acetic acid. Treatment should be continued until the sections are a light reddish-violet color. It is preferable, however, to tone the sections for an hour in a very dilute solution of gold chloride, using 2 to 3 drops of 1 per cent gold chloride in 10 c.c. of distilled water. The 1 per cent gold chloride solution keeps for long periods in the dark.

7. Wash in distilled water.

8. Fix in 5 per cent sodium thiosulfate for one-half to one minute.

9. Wash freely in distilled water. Mount on glass slide from 50 per cent alcohol, blot carefully, complete dehydration in two changes of absolute alcohol, and clear in carboxylol (1:10). Mount in balsam.

There is excellent staining of the axis cylinders and dendrites as well as the intracellular neurofibrillar network. The latter appears as an interlacing web of delicate fibrils, dark brown or black, depending upon the degree of toning in the gold bath. The axis cylinders are stained an intense black, as are the dendrites. There is occasional faint impregnation of the glial cells and in tissue, which is the seat of marked gliosis, impregnation of the glial fibers may be quite definite. The background is colorless or light purple, depending upon the toning.

The silver nitrate solution should be made fresh just before use. Care should be taken in preparing the silver bath that an excess of ammonium hydroxide is not added. A small amount of very fine granular, black precipitate should be allowed to remain undissolved. At least a liter of double distilled water should be used in washing, decanting the fluid after allowing the precipitate to settle. The formalin used for reduction should be neutralized with calcium carbonate. Sections may be carried up to the point of

toning and then counterstained with the Alzheimer-Mann method for glial fibers. Herxheimer's method for fat is also a very useful counterstain.

Cajal's Method for Neurofibrils.—The following method of Cajal is taken from Spielmeyer and is said to give excellent impregnation of neurofibrils in the cerebellum. It has been found quite useful for tumor material.

1. Frozen sections, cut at 30 to 40 microns, from formalin-fixed tissue are washed rapidly in two changes of distilled water.

2. Place sections for two to twelve hours at room temperature in the following mixture:

2% silver nitrate	10 c.c.
Pyridine	8 drops
96% alcohol	5 to 6 c.c.

The sections are removed when they assume a light brown color. Placing them in an incubator hastens the reaction and assures better impregnation. Three to five hours is usually enough time.

3. Pass the sections rapidly through absolute alcohol.

4. Reduce without previous washing in the following solution:

Hydroquinone	0.3 gm.
Distilled water	70.0 c.c.
Neutral formalin	20.0 c.c.
Acetone	15.0 c.c.

The reduction is completed in one to three minutes.

5. Wash freely in distilled water.

6. Tone in gold chloride 1:500; wash in distilled water and fix in 5 per cent sodium thiosulfate for one to two minutes.

7. Wash thoroughly in distilled water. Mount on a glass slide from 50 per cent alcohol, blot carefully, complete dehydration in two changes of absolute alcohol, and clear in xylol. Mount in balsam.

The stain may be completed after reduction, washing freely in distilled water, fixing in 5 per cent sodium thiosulfate, washing, dehydrating, clearing and mounting as usual. This gives the neurofibrils a deep brown to black color against a golden-brown background.

There is intense impregnation of the nonmyelinated axis cylinders, especially in the cerebellum where the arborizations of the basket cells and the mossy and climbing fibers are clearly shown. These elements are stained black upon a light gray or purplish-gray background, the latter if gold chloride toning is employed after reduction.

The reducing solution keeps well in a tightly stoppered bottle. Passage of the sections through the absolute alcohol after impregnation in the silver bath should be done rapidly to prevent loss of silver. The method gives good results on tissue which has been fixed in formalin for several years.

(To be continued.)

A SIMPLE BLOOD PIPETTE CLEANER*

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IN AN effort to lessen the drudgery of a busy clinical laboratory, the following device for simultaneously cleaning several blood pipettes was made.

Fig. 1.

A wide-mouthed (60-70 c.c. to one-half pint) jar is fitted with a rubber stopper through which eight small (3 mm.) holes in two straight lines, and one larger (6 mm.) hole near the edge of the stopper are bored, as shown in the drawing. Into the larger hole is fitted a glass L-tube connected by a rubber tube to a faucet suction pump. The eight holes, which are sloped toward one another on the outside of the bottle, receive the butt ends of the pipettes.

*From the Department of Pathology, University of Louisville, Louisville, Ky.
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In use, the holes are loaded with pipettes, and the suction is turned on. The bottle is then inverted successively over the distilled water, alcohol, and ether bottles, so that the tips of the pipettes dip into these fluids. Waste fluid entering the wash bottle is drawn off by the suction tube, whose inner end lies flush with the inner surface of the stopper and is held downmost in use. If all eight holes are not needed, the unoccupied ones can be covered by the finger.

The pipettes are dried by merely setting the bottle down on the table, the hands then being free for other tasks. If one wishes, the ether wash can be omitted, and the drying done for several minutes after the alcohol.

SMALL ANIMAL HOLDERS FOR INTRAVENOUS INJECTION*

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INTRAVENOUS injections in rodents are no novelty to pharmacologists and physiologists, but satisfactory animal holders seem to be wanting. In the late Professor A. S. Loevenhart's laboratory at the University of Wisconsin, a mailing case was used for injections into one of the tail veins of rats—the bottom of the case being perforated, and the lid being replaced by a cork with a hole at the edge to accommodate the tail. This simple device usually served the purpose. Its chief disadvantages are, however, retraction of the tail by small animals and difficulty in cleaning the cardboard case. With the aim of improving this apparatus, we have devised metal holders† for mice, rats, and guinea pigs, and found them so convenient that they may be suitable for other investigators.

The rat holder as shown in Fig. 1 consists of two hollow cylinders, one inside the other. The outer cylinder measures 9.4 cm. in length, and 5.2 cm. in outside diameter, its thickness being 0.25 cm. all around. One end of it is closed with a perforated plate. The holes are evenly distributed, 0.65 cm. in diameter, and 7 in number—adequate for ventilating purposes. The inner cylinder is telescoped into the outer one. It is 7.5 cm. long, 0.2 cm. thick, and 4.8 cm. in outside diameter. A swinging door with a marginal hole, 0.9 cm. in diameter, for the tail, is fixed around a pin. A shallow groove is cut longitudinally on the outer surface of the inner cylinder to slide over the tip of screw *A*. If the rat is heavy, the diaphragm *B* is swung out around the pin to allow more space. This has the same number of perforations as the end plate. The entire holder is mounted on a solid, short rod, 6.3 cm. long and 1.25 cm. in diameter, and is fitted into a hollow tube rooted in the center of an iron stand, 15.4 cm. by 12.9 cm. by 2.5 cm. (height). It can be lowered or raised by means of screw *C*.

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*From the Lilly Research Laboratories and Indiana University School of Medicine, Indianapolis.

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†Those workers who do not have shop facilities may communicate with Mr. Elmer Lee, a machinist, 5040 West Washington Street, Indianapolis, concerning the supply of these holders.

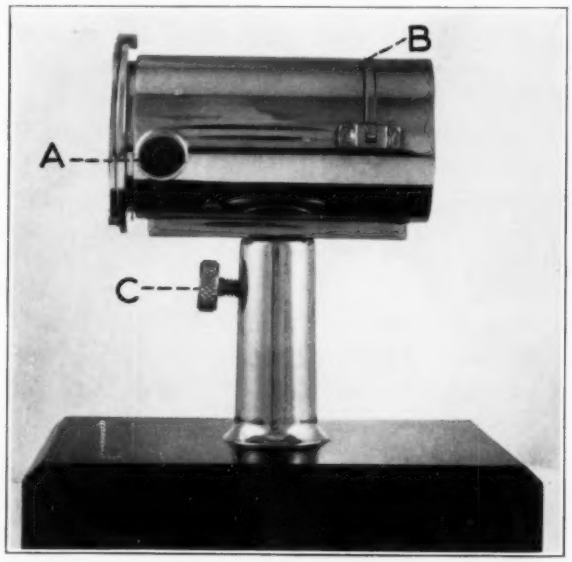

Fig. 1.—A rat holder.

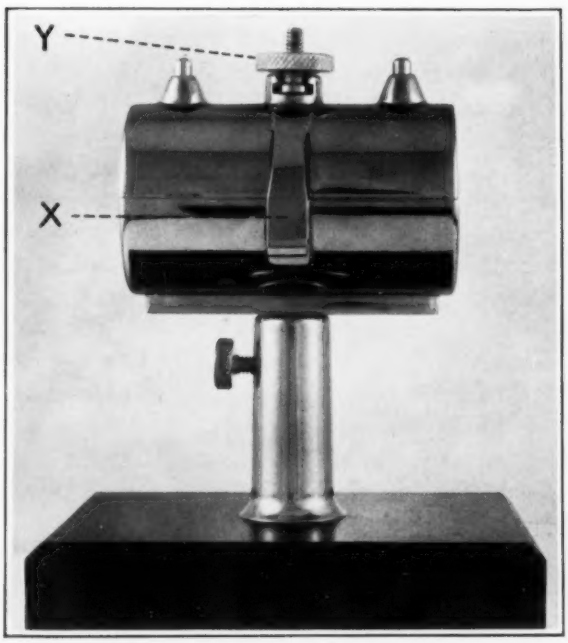

Fig. 2.—A guinea pig holder.

To prepare for an intravenous injection, the gate is left open, the inner cylinder is pulled out four-fifths or two-thirds of its length, and the rat is allowed to walk into the chamber. After the gate is closed leaving the tail outside, the inner cylinder is pushed as far as possible, without overcrowding the animal, and is fastened by screw *A*.

The mouse holder is similar to that for the rat, only much smaller. The outer cylinder is 4.1 cm. long, 0.2 cm. thick, and 3 cm. in outer diameter. The end plate has 16 holes, each measuring 0.32 cm. in diameter. The inner cylinder is 4.1 cm. long, 0.2 cm. thick, and 2.7 cm. in diameter. The marginal hole for the tail is 0.38 cm. in diameter. No diaphragm is needed on the outer cylinder because the holder can easily accommodate mice of all sizes.

The cutaneous veins of the four legs of guinea pigs offer good routes for injections, the forelegs being more preferable than the hind limbs, and the left foreleg more preferable than the right for a right-handed individual. The holder as shown in Fig. 2 will support the body of the animal with the head and forelegs exposed at one end, and the hindlegs at the other. This bivalvular device, 10.4 cm. long, opens around the hinges, and locks itself by a spring catch *X* when the upper half is approximated with the lower half. A thin strip of steel sheet is soldered below the lower border so that the upper border can overlap it in closing. This prevents catching of hair or skin between the two halves. The lower half is also provided with semilunar borders at both ends. In order to hold the pig tightly, a concave plate is hung on the inside of the upper half and can be lowered or raised by screw *Y*.

The advantages of these holders are obvious: simplicity of manipulation, complete immobilization of animals, speeding up of the work, capability of the operator to work alone, and ease in cleaning. It cannot be overemphasized that the toxicity data obtained by the intravenous injection in small warm-blooded animals are far more valuable than those by subcutaneous injection in amphibians, such as the frog. Incidentally, the same holders can also serve to secure blood samples, either for cell counting or for microchemical analyses. The exposure of guinea pigs' heads permits the measurement of their pupils, teeth, and so forth. The sizes of these holders are entirely arbitrary to suit our own needs. The mouse holder will accommodate animals weighing between 14 and 35 gm.; the rat holder, between 80 and 200 gm.; and the guinea pig holder, between 250 and 350 gm. The material employed for making our apparatus consists of cast iron and stainless steel. The finished product is all chromium plated, except the base which is painted black. There is no reason, however, why other metals or hard rubber cannot be tried.

SUMMARY

Holders for mice, rats, and guinea pigs for intravenous injection have been described.

A SIMPLE METHOD FOR VISUALIZING PRESSURE AND VOLUME CHANGES APPLICABLE TO OBSERVATIONS ON THE RHYTHMIC VARIATIONS IN THE PERIPHERAL CIRCULATION*

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INCIDENTAL to a survey of the methods used in the study of circulation among chronic arthritics[1] it occurred to me that it might be possible to employ a simple device utilizing the elastic properties of thin glass plates, together with the phenomenon of Newton's rings in detecting the changes of volume and pressure associated with the peripheral flow of blood. As far as I am aware, no method employing this principle has hitherto been described.

To test the practicability of such a procedure, an ordinary "noncorrosive" microscopic slide (25 by 75 by 1 mm.) and a No. 1 cover glass (22 by 40 mm.) were cleaned with chromic acid solution, rinsed with water, dried, and then flamed to remove a film of moisture. This preparation simply served to provide fairly clean and relatively nonsticking surfaces. The two glasses were pressed together with a shearing motion. One edge of the cover glass was then attached to the slide near the midline by means of cement or a narrow strip of adhesive tape or of gummed paper. When such a slide is held in a rigidly fixed position and pressure or a weight is applied to the cover glass, a pattern of light and dark bands appears. This optical phenomenon, due to the interference of light waves associated with reflecting surfaces separated by a thin layer of air of nonuniform thickness, is known as Newton's rings. When viewed from the angle of reflection in reflected polychromatic light, the pattern appears as a series of brilliantly colored bands against a white background. When viewed in monochromatic light, the bands are made more clearly evident and appear as dark lines against a lighter background of the color used. The latter arrangement is preferable for observations on peripheral pulsation inasmuch as bright reflected light obscures the pale colored bands which first appear under minimal pressure.

These phenomena have been used in static measurements of the curvature of lenses, and in the determination of the optical regularity of plane surfaces. The proposed use involves the additional utilization of dynamic phenomena which take advantage of the fact that when the pressure applied to the thin deformable glass slip is varied, the thickness of the air layer is thereby modified, and this in turn alters the interference pattern. When changes of pressure are exerted in a regular sequence, the impression of a continuous movement of bands is produced which parallels the modification of applied pressure.

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Thus, if such an arrangement of glass plates held in one hand is placed over the wrist with one end of the slide resting on the styloid process of the radius with the cover slip directed downward over the radial artery while slight, but constant, pressure is maintained from the opposite end of the slide, pulsations are made clearly evident by a rhythmic shifting of the colored bands. By fixing the attention on one band or one central circular area, it is possible to recognize a rapid extension of a band from its base line or an enlargement of the circle succeeded by a decrease in diameter, and this in turn is followed shortly by a slight rebound which is succeeded by a return to the original position. The phases of the pulse are thus made visually evident by the momentary "peaks" in the alternating ebb and flow of the bands from their basal levels.

The circulatory pulsations in the fingers may be made evident in a similar fashion. In order to maintain the finger in a fixed position and to apply constant pressure on the slide it is necessary to provide a stage for supporting the finger and a holder for the slide. A simple, but adequate, rack for this purpose has been made with a ring stand, clamps, and a burette holder, arranged so that the finger may be rested upon a block and the slide, held in a burette holder, may be turned downward to bring the slide to a position in which the lower surface of the cover slip touches the finger. If a stand is not available, a substitute may be made by laying the slide on a finger and loosely attaching it by means of strips of adhesive tape to a splint on the opposite side of the finger. The optimal position is that in which the minimal degree of pressure required for producing bands is exerted. Under these conditions pulsations become clearly evident. Obstruction of blood flow to the hand by the application of pressure on the brachial artery results in a prompt cessation of the "movement" of the bands.

Fig. 1 shows the general appearance of the interference bands produced by application of the slide to the finger. Illumination, daylight; exposure, one-tenth second; size, one-half actual.

Fig. 2 illustrates the general sequence of changes which can be recognized readily with the simple device applied over the radial artery or to the finger of a normal subject. It is evident from this diagram that a marked amplification of the actual vertical movement, with respect to the plane of the glass surface imparted to the thin glass, is achieved by following the horizontal movement of the interference band. If it can be assumed that the perceptible change of color, representing the modification in thickness of the layer of air, is indicated by the respective wave lengths of light, it would follow that a movement imparted to the thin glass in the order of 0.0001 mm. is recognizable. Since with such a color change there is a shift of the boundary band equivalent to 5.0 mm., it may be deduced that an apparent amplification factor approaching 50,000 times the actual movement may be realized.

With ordinary cover slips the pattern produced by the lines in the colored field is not uniform, due to irregularities in the surface of the glass. With cover slips, such as are used for blood counting chambers, the bands appear

in a more nearly uniform arrangement. The latter combination while presenting wide bands and a clear-cut regular pattern is not essential for recognizing changes in the magnitude of applied pressure. In fact, the thicker glass is less sensitive as an indicator of rapid changes and shows a definite lag in following the alternations of applied pressure. In addition, the forces required to modify the pattern are greater than with thinner glass because of the lesser degree of deformability of the thicker glass.

Fig. 1.—Photograph of sphygmoscope showing interference bands.

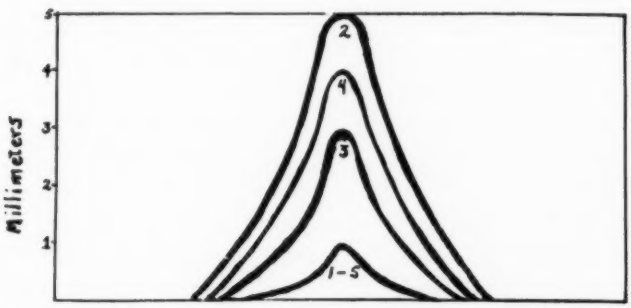

Fig. 2.—Diagram showing the relative position of an interference band at intervals during a single pulse cycle.

Position	Time Seconds Approximate
1	0.0
2	0.2
3	0.3
4	0.4
5	0.5 to 1.0

In order to increase the sensitivity to the rapid alternation in pressure and provide a fairly reproducible pattern, a segment of a No. 1 cover slip, cut to 8 mm. by 22 mm., was attached to a slide permitting space at each end for clamps. It has also been noted that slight elevation of one or both ends of the cover slip by means of a narrow thin strip of glass or thin copper foil (0.002 inch thick) makes the arrangement more rapidly responsive to changes in applied pressure. This is due to the fact that with the release of pressure from the center of the thin glass there is as light, but definite, tendency for the glass to spring back beyond the limit necessary for the appearance of interference bands.

There are several obvious ways in which such a device may be utilized clinically. It might be employed for the purposes served by other forms of oscillometric and sphygmoscopic devices. Among these may be mentioned the detection of the presence or absence of pulsations in a member suspected of having an obstruction, as an oscillometer. In addition to this use, the device may be employed as a sphygmoscope or an indicator for determining the pres-

ence of conditions associated with abnormalities of the pulse pattern, e.g., in showing the relative position of the dicrotic wave in aortic regurgitation and other cardiac states.

To survey further the practicability of the procedure, a preliminary series of observations upon a few normal individuals and several patients with clear-cut cardiac and circulatory diseases have been made. Differences in the pulse pattern have been made qualitatively evident. In cases with aortic regurgitation the dicrotic notch has been recognized as being closer to the base line than in normal subjects. In patients with impaired circulation in the hands and feet absence or decrease of pulsations has been made evident. These observations indicate that the principle may be utilized as a supplement to other methods for the ready demonstration of variations in the peripheral circulation.

The procedure is now presented merely as an accessory method for visualizing the state of circulation at the periphery. Pulsations which are made evident at peripheral points are obviously the resultant of those forces driving the blood through these parts and the elasticity of the tissues. As described, the procedure apparently provides an index of the sudden rhythmic volume changes in the peripheral tissues chiefly determined by the cardiac cycle, and represents roughly the pulse volume changes.

One evident shortcoming inherent in the simplicity of this procedure is that it is not strictly quantitative and includes no method for providing a permanent record of the data for comparative purposes. Refinement with respect to these factors by employing optically plane, thin glass slips, a mechanical stage suitable for adapting the glass at a constant and reproducible pressure, and elaboration with photokymographic accessories for recording the ebb and flow of the interference bands, would meet these difficulties and make possible a better analysis of the pulsations. However, the simple procedure described provides a new and qualitatively adequate means for visualizing the presence of pulsations, together with some of the characteristics of the rhythmic pressure and volume changes in the peripheral tissues.

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UNRELIABILITY OF OXALATED BLOOD IN DETERMINATION OF ERYTHROCYTE SEDIMENTATION RATE*

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SINCE its rediscovery by Fahraeus in 1918,[1] the suspension stability of plasma, better known as the sedimentation reaction of erythrocytes (designated S. R.), has been the subject of numerous laboratory and clinical studies aiming to determine its value as a diagnostic and prognostic procedure. The recorded findings of various workers are so confusing and contradictory that the relationship of the sedimentation rate to the condition of the patient is still unsettled. It was simple enough, in the days of limited medical knowledge, to draw fundamental conclusions from the manner in which large quantities of drawn blood settled into layers; but modern medicine insists on a more detailed study of the falling rate and its relationship to morbid processes. Various techniques have been suggested, all depending on the use of chemical anticoagulants. This is merely one of a number of maneuvers likely to introduce errors in the final determination, errors which internists, although cautioned about, have not fully appreciated, especially as regards the anticoagulant. Too often variations due to technical factors have been assumed to be due to disease processes, and undoubtedly this is partly responsible for the contradictory opinions concerning the test. All anticoagulants introduce some degree of error, but in general wider variations have been noted with oxalates than with the others. This is especially important in view of the growing practice of hospital laboratories of using oxalated blood for many routine determinations according to systems devised by hematologists.[2] The tests that are said to give reliable results are the red and white cell counts, platelet and reticulocyte counts, hemoglobin, chemical contents, and icteric index if determined within three hours, and also the erythrocyte volume index and the sedimentation rate if the blood is mixed in the proportion of 1 c.c. per 2 mg. potassium oxalate. The adoption of a system of this kind saves considerable time and trouble of patient and intern, inasmuch as a single bleeding gives the laboratory opportunity to complete the routine determinations now expected of all new admissions to hospitals. This is the main reason for the substitution of oxalate for citrate in determining the sedimentation rate. Another is that dry oxalate can be kept indefinitely in a container ready for use, whereas citrate solutions tend to deteriorate and must be kept sterile if drawn into the syringe when obtaining the blood. Furthermore, the method has been recommended as reliable by good authorities. At one time the author included the sedimentation rate test in routine determinations on oxalated blood from tuberculous patients, but found

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the results to be so variable that its dependability as an index of the status and progress of lesions was questioned. By exclusion, the anticoagulant was suspected to be the cause of discrepancies, and, in order to check on this, several experiments were performed, the results of which it is felt deserve recording.

Heparin and hirudin are considered to be the best anticoagulants in that they have very little or no influence on the sedimentation rate, but their expense and uncertainty preclude their routine use. Fluorides may be dismissed from consideration. Up to recent years the most popular method was that of Westergren,[3] in which blood is mixed with citrate solution. As for oxalates, some internists drew attention to undependable results following their use; others published data showing quite consistent readings when the tests were run in duplicate with citrated or heparinized blood. That the question of the influence of various anticoagulants on the sedimentation rate is in need of further study is quite evident from the contradictory conclusions arrived at by various workers, several of whom will be quoted here.

Westergren[4] found that the sedimentation rate of blood citrated according to his technique is not materially affected, but that it is slowed after twenty-four hours. Peterman and Seeger[5] noted variations among different anticoagulants which were difficult to interpret. Pinner, Knowlten, and Kelly[6] ran a series of duplicate tests using citrated and oxalated blood according to Westergren's method (height of column), and found that low sedimentation rates are usually higher in oxalated blood than in citrated blood, and that with high rates the relation is frequently reversed. Ernstene[7] claims that dilution with sodium citrate solution makes accurate readings impossible. Rourke and Plass[8] found that sodium fluoride, potassium oxalate, and potassium citrate retard the falling rate; that the rate of sedimentation varies inversely with the concentration of salt necessary to prevent coagulation; and that increasing the concentration of any of the above anticoagulants always slows the rate. Contrariwise, Wintrobe and Landsberg[9] claim that potassium oxalate in the proportion of 2 mg. per 1 c.c. of blood is a satisfactory anticoagulant and does not depress the rate of settling. They remark that the disagreement between their observations and those of Rourke and Plass can probably be accounted for in part by the somewhat lower concentration of oxalate they used, and then add that they failed to note a consistently depressing effect on sedimentation even with greater quantities of oxalate such as these investigators used. Hinton[10] claims that citrate and oxalate slow the rate by altering the shape and surfaces of the red blood cells and so diminish the agglutinability of the cells. Sasano, Ordway, and Medlar[11] found that oxalates in minimal quantities are very satisfactory in sedimentation rate determinations, yet Table VI in their article shows the average rate of ten cases run in duplicate to be 11.2 mm. by the citrate method and 17.3 mm. by the oxalate method, results which may be satisfactory in that they both are slow but certainly not accurate for comparative purposes. Dorfman and Brooks[12] obtained results which show: first, delay in performing the test causes slowing of the sedimentation rate with both oxalated and heparinized blood; second, the effects of delay are shown much earlier with oxalated blood than with heparinized blood; and third, heparinized blood settles more slowly than oxalated blood. Barnick,

Gregg, and Guernsey[13] express satisfaction with citrate used according to Westergren's method and with oxalate in the proportion already mentioned. Ham,[14] however, thinks that solutions of sodium citrate cause significant slowing of the sedimentation rate due to dilution and to salt effect, and that potassium oxalate in a concentration of 2 mg. per 1 c.c. of blood has no significant effect. Haskins, Trotman, Osgood, and Mathieu[15] state that comparative studies of oxalated blood and blood diluted with citrate showed that the use of oxalate did not introduce clinical error; the test is to be started within three hours of drawing the blood. Mattice[16] found dry oxalate superior to citrate solutions, but does not concur with Haskins and Wintrobe in the belief that the test may be set up within three hours of taking the blood; it must be done immediately. At room temperature pathologic blood rapidly changes its sedimentation rate in the direction of normal. Whitby and Britton[17] believe the test to be so sensitive that an exact technique is essential. It is important to set up the blood at the earliest possible moment; results after an hour or two delay are never so reliable as those obtained immediately.

Obviously, such divergent opinions must mean that the test is not as free from intrinsic faults as its technical simplicity suggests. Presumably, certain very important precautions were observed by all those whose opinions were quoted, such as the use of clean glassware and the maintenance of tubes in a perfectly vertical position during the test. Some of the articles did not mention the length of blood column or time intervening between obtaining the blood and performing the test. Because all these are potential sources of error, and because comparison of results obtained by different investigators is unfair unless a uniform technique is used, the procedure followed by us will be given in some detail.

Blood was withdrawn from a vein in the cubital fossa with a dry syringe before breakfast. Citrated specimens were prepared by mixing 4 parts of blood with 1 part of 3.8 per cent sodium citrate solution already accurately measured out in a container. Oxalated specimens were prepared by mixing blood with dry potassium oxalate in the proportion of 1 c.c. to 2 mg.; in practice, 8 c.c. of blood were transferred to a container in which 0.8 c.c. of 2 per cent potassium oxalate solution had been dried in a hot air oven. The specimens were then drawn up to the 200 mm. mark in tubes having a bore of about 2.5 to 3 mm., and the tubes were set up in a rack in a perfectly vertical position. The tests were performed at room temperature within a half hour of obtaining the specimens. The tubes were cleaned with tap water, alcohol, and ether, and dried with a vacuum pump. The column of clear plasma was read at the end of fifteen, thirty, forty-five, and sixty minutes, but *only the final readings are recorded in the tables.* The tubes were read in succession at intervals of twenty seconds, the approximate time taken to set up each one in order. This precaution must be observed for exact results when performing the test simultaneously with many tubes, especially when the rates are fast. Patients are designated alphabetically, the letters representing different patients in each experiment.

To Note the Consistency of S. R. With Citrated Blood.—Sufficient blood was withdrawn from each patient to set up 4 columns of blood simultaneously, and

the rates were determined at once. It will be noted from Table I that the fall is quite consistent. This serves as a check not only on the consistency of the sedimentation rate of citrated blood but also on the accuracy of our technique.

TABLE I

	TUBES			
CASE	1	2	3	4
A	19	19	19	17
B	22	24	27	26
C	39	34	37	42
D	73	71	65	70
E	55	55	54	53
F	7	6	8	7
G	42	40	41	42
H	14	14	14	13

To Note the Consistency of S. R. With Oxalated Blood.—The above experiment was repeated, using oxalated blood. Table II shows that the fall is quite consistent if the rates are determined without delay.

TABLE II

	TUBES			
CASE	1	2	3	4
A	20	20	18	19
B	32	32	31	30
C	46	43	44	45
D	8	8	7	7
E	105	104	101	103
F	129	127	130	128
G	3	3	3	3
H	18	17	19	18

To Note Effect of Time Interval on Consistency of S. R. With Oxalated Blood.—Sufficient blood was drawn and citrated in the manner described to determine the sedimentation rate immediately, and also one, two, and three hours later. Table III shows that the rates are consistent even after an interval of three hours.

TABLE III

CASE	STAT	1 HR.	2 HR.	3 HR.
A	112	105	105	108
B	110	108	109	101
C	35	35	31	32
D	20	24	14	17
E	43	49	43	40
F	6	3	5	4

To Note Effect of Time Interval on Consistency of S. R. With Oxalated Blood.—The above experiment was repeated, using oxalated blood. Table IV shows that the greater the time interval between oxalating the blood and determining the sedimentation rate, the greater and more frequent is the retardation in sedimentation.

TABLE IV

CASE	STAT	1 HR.	2 HR.	3 HR.
A	74	78	58	46
B	68	29	15	6
C	17	7	5	2
D	25	15	8	4
E	130	120	110	105

To Note Effect of Time Interval on S. R., Using Citrated and Oxalated Blood in Duplicate Tests.—Blood was drawn from 19 patients and each sample was divided into two portions; one was citrated and the other was oxalated as described. The sedimentation rates were determined on all specimens soon after withdrawal. In 5 cases the rates were determined again two hours later; in 5 other cases, three hours later; and in 9 cases, four hours later. The results are shown in Table V.

TABLE V

CASE	SOON AFTER BLEEDING		2 HR. LATER	
	CITRATED	OXALATED	CITRATED	OXALATED
A	80	88	89	65
B	63	53	57	12
C	60	32	75	24
D	16	36	16	11
E	78	100	78	77
			3 hr. later	
F	60	78	65	7
G	33	39	32	5
H	53	48	52	9
I	92	89	95	6
J	99	113	122	7
			4 hr. later	
K	34	36	32	70
L	41	43	37	11
M	44	42	43	45
N	16	18	12	7
O	42	47	34	9
P	120	125	97	16
Q	30	32	28	31
R	56	59	52	22
S	9	11	4	2

On comparing the figures in columns 1 and 2 (citrate and oxalate soon after withdrawal of blood), one will note that there is a rather marked discrepancy in cases C and D, in one case the oxalated specimen showing a rate twice that of the citrated specimen, and in the other case just the reverse. In all other cases the differences, though moderate and not of great practical significance, are still too large for comparative purposes. When tested immediately, a citrated blood will usually show a faster rate than the same blood oxalated; occasionally the reverse occurs. After an hour or more, the rates are markedly retarded with oxalated blood.

The figures in columns 1 and 3 (citrated blood tested soon after bleeding, and two, three, and four hours later), bear out what was seen in Table I: that citrated specimens give readings that are quite consistent over an interval of three hours, and even of four hours.

Comparison of the figures in columns 1 and 4 (citrated blood soon after bleeding, and oxalated blood two, three, and four hours later), shows that these intervals of time give sedimentation rate readings which are ridiculously retarded in 11 out of 19 cases, and markedly increased in one case. It is quite apparent that any material interval of time between oxalating the blood and determining the sedimentation rate makes this procedure very untrustworthy.

To Note the Effect on the S. R. of Adding Oxalate to Citrated Blood.—Blood was withdrawn from 9 patients and citrated in the usual manner. Each specimen was then divided into three portions of 3 c.c. each. One portion was left as such; to the second portion was added 1 drop of a saturated solution of sodium citrate; to the third portion was added 1 drop of a saturated solution of potassium oxalate. All three specimens from each patient were then set up and the sedimentation rate determined simultaneously. It will be noted from Table VI that the increased concentration of citrate causes a decrease in the sedimentation rate rather consistently, as expected. When oxalate is added, however, the changes are inconsistent, the rates being much faster in 1 case, much slower in 4 cases, and approximately unchanged in 4 cases. It is evident that oxalate introduces a variable that makes it absolutely unreliable as an anticoagulant in determining the sedimentation rate. Of course, in actual practice much smaller quantities of oxalate are used in performing the test; but, as was noted in Table V, even then the results are undependable soon after mixing, much more so with increasing intervals of time.

TABLE VI

CASE	ORIGINAL	CITRATED	OXALATED
A	27	24	62
B	113	86	12
C	31	26	8
D	22	20	25
E	36	34	38
F	45	40	18
G	12	8	5
H	3	2	1
I	32	26	6

To Note Whether Diluted Oxalated Blood Reduces the Error in the S. R.—It has been shown that blood diluted up to 20 per cent of its volume with 3.8 per cent sodium citrate solution has a sedimentation rate not much different from the same specimen prepared with hirudin or heparin; the resulting increase in rate, if any, certainly does not approach that expected according to results obtained in experiments in which the volume index of blood was decreased by dilution with its own plasma.[10] The question arose as to whether dilution had any tendency to correct the error introduced by an anticoagulant. Accordingly, the following experiment was performed. Blood was citrated with citrate solution by Westergren's technique, and another portion of the same blood was mixed with dry potassium oxalate. The latter was divided into two portions, one of which was then diluted to one-fifth of its volume with oxalated saline (0.2 gm. potassium oxalate per 100 c.c. normal saline). The use of pure normal saline dilutes the anticoagulant effect of the original oxalate, hence the necessity for additional oxalate in the diluent to prevent clotting. The three specimens were set up at once and the sedimentation rate determined.

From the results in Table VII, it will be seen that the sedimentation rates of the diluted oxalated blood (termed wet oxalate) agree more closely with those of citrated blood than do the dry oxalate specimens. Hence, if it is desired to do routine sedimentation rate determinations on specimens of blood prepared with

dry oxalate in the routine way, less aberrant results may be expected if the portion of blood used for this purpose is first mixed with one-fifth its volume of oxalated saline, provided there is little delay in the procedure.

TABLE VII

CASE	CITRATED	DRY OXALATED	WET OXALATED
A	6	6	6
B	7	8	6
C	13	20	10
D	21	26	20
E	5	12	9
F	14	25	15
G	85	86	68
H	118	122	128
I	48	63	64
J	62	84	64
K	11	13	11
L	15	26	17
M	101	122	112
N	116	116	101
O	32	12	26
P	90	120	99
Q	88	117	90
R	66	99	64
S	61	90	61
T	37	56	38
U	74	89	75
V	4	6	4
W	71	92	72
X	105	101	107
Y	51	90	56
Z	80	110	80

SUMMARY AND CONCLUSIONS

1. Blood citrated according to Westergren's method shows sedimentation rates which maintain consistent rates for at least four hours. This consistency warrants the acceptance of the sedimentation rate with citrated blood as reliable; any deviation from the true rate due to dilution or salt effect is constant, and has already been discounted in the establishment of the normal rates by this method.

2. Blood mixed with dry oxalate in minimal quantities, i.e., 2 mg. of the potassium salt per 1 c.c. of blood, will show sedimentation rates which are fairly reliable only if performed with no delay.

3. More accurate sedimentation rates will result with blood thus oxalated for routine studies if the portion used for the test is diluted with one-fifth its volume of oxalated normal saline and tested at once.

4. Sedimentation rate determinations made with oxalated blood are absolutely unreliable if the delay between oxalating and testing is an hour or longer; the longer the delay, the greater the incidence and degree of error.

5. In large institutions where it is desirable to include the sedimentation rate in the routine blood determinations and heavy laboratory schedules make immediate examination impractical, more reliable and accurate results will be obtained by distributing tubes containing 0.5 c.c. of 3.8 per cent sodium citrate solution and having 2 c.c. of blood syringed into this container at the time blood

is drawn for other determinations. This amount (2.5 c.c.) suffices for use in a Westergren tube (200 mm. by 2.5 mm. column). The test may be performed at any time within four hours of citration.

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LABORATORY METHODS FOR TESTING FUNGICIDES USED IN THE TREATMENT OF EPIDERMOPHYTOSIS*

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THE disease condition recognized as epidermophytosis, commonly known as "athlete's foot," has increased considerably during recent years. This skin infection now affects approximately one-third of the population and is widely and generally distributed throughout most parts of the world. With the increased prevalence of this disease and more general knowledge as to its cause, there has been a consequent increase in the use of certain fungicides in treating this condition. The fungicides employed have been selected after clinical experiences have demonstrated their merit for this purpose. In vitro tests have also been made to some extent in which various laboratory procedures have been employed. So far, however, none of the laboratory methods suggested have proved entirely satisfactory.

Probably the first laboratory method of merit was the one proposed by Schamberg and Kolmer[1] in 1922. In this method three of the most common pathogenic fungi were used as test organisms for determining both fungistatic and fungicidal activity. For determining fungistatic activity the test organisms were inoculated on Sabouraud's agar slants containing various concentrations of the compounds tested and then observed for inhibition of growth. In the fungicidal test the same test organisms were grown on Sabouraud's agar, suspended in saline solution, and mixed with different concentrations of the fungicides tested, transfers being made to slants of Sabouraud's agar at time intervals from fifteen minutes to twenty-four hours.

This method, or some modification of it, has been used by many other workers in this field and is still quite generally employed. Myers and Thienes[2] used essentially the same method in reporting on the fungicidal activity of certain volatile oils; Kingery and Adkisson,[3] Woodward, Kingery, and Williams,[4, 5] and Kingery, Williams, and Woodward,[6] used certain modifications of the method of Schamberg and Kolmer in their extensive studies on volatile oils, stearoptens, and phenol derivatives. Klarmann, Shternov, and Gates[7, 8] made use of essentially the same technique in their studies on the fungicidal activity of derivatives of parachlorphenol and orthochlorphenol. Stovall, Pessin, and Almon,[9] and Maplestone and Dey[10] used modifications of this test in studies on thymol and certain other fungicidal substances. McCrea[11] in 1931 suggested that the Schamberg and Kolmer method, or a slight modification of it, be adopted as the standard method for testing fungicides.

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While this method of test has served a useful purpose in separating those substances which are fungicidal and may be effective for treating epidermophytosis from those which may not have any merit for this purpose, there are, however, certain objections to this method which should be mentioned at this time, especially since this procedure has been suggested as a standard test. The first objection to this test is that it does not in any way simulate practical conditions of use. In fact, the conditions created in this test are just opposite to those met with in practice. Suspending the organisms and spores in saline and shaking with glass beads to break up the clumps and matted mycelium before exposing to the action of the fungicide is quite different from the condition of the organisms as they occur in infected skin. Under the conditions of actual infection the organisms are closely matted and embedded in tissue. Testing the killing power of fungicides after breaking up the matted mycelium and spores of the test organisms is not logical and does not simulate practical conditions in the least. Also, no clinical evidence has been offered to prove that this arbitrary in vitro test is a satisfactory means of testing such fungicides. Since the method is an arbitrary laboratory test, there should be supporting clinical tests to show that fungicides passing this test will be effective in practical use and that those not passing it will be ineffective in clinical practice.

Another serious objection to the method of Schamberg and Kolmer as described by them is that in transferring from the mixture of fungicide and culture no precautions are taken to prevent inhibition of growth on the subculture slants. The method specifies that several loopfuls of the fungicide-culture mixture are transferred directly to slants of Sabouraud's agar. Since enough of the fungicide may be and probably is carried over to the medium to exert a fungistatic effect, false readings are likely to be made, and inhibition of growth may be interpreted as actual killing. These defects in this method are such that the test in its present form should not be used for testing fungicides which are intended for use in treating epidermophytosis.

Another method for testing fungicides that has been used by certain workers is the phenol coefficient test. Emmons[12] was among the first to use this technique in testing the fungicidal activity of some common disinfectants. The highest dilutions of disinfectants which killed the fungi used as test organisms in five minutes and thirty minutes were divided by the highest dilutions of phenol accomplishing the same result, giving a figure which was designated as the phenol coefficient. Similar technique was employed by Gomez-Vega,[13] Hasseltine and Hopkins,[14] and Dunn.[15]

The phenol coefficient test does not lend itself well to the testing of fungicidal activity against fungi other than bacteria. The method is designed for the use of bacteria as test organisms and is not suited to the use of those fungi which cause epidermophytosis. The medium employed is not suitable for normal growth of these fungi; it is difficult to secure uniform distribution of the organisms in broth; the amount of fungicide-culture mixture transferred is too small; the test is not sufficiently severe and comparison with phenol is not necessary nor even desirable. Also, no information is obtained as to the penetrating properties of

the fungicides tested. For these and other obvious reasons the phenol coefficient method is not suitable for testing fungicides.

Other methods of testing fungicides have been suggested from time to time during the past few years. Strickler[16] used collodion sacs containing suspensions of fungi for testing the fungicidal activity of elemental iodine. This method is well suited for the purpose intended but would not be applicable as a method for testing other kinds of fungicides. Sharlit and Muskatblit[17] and Sharlit[18] used what they have designated as the "membrane method" which is best suited for testing volatile substances, such as thymol, etc., and water soluble fungistatic compounds. The fungicide tested is incorporated into collodion which is then spread inside test tubes into which the agar medium is slanted. The test organisms are then inoculated on the surface of the slants and incubated. If the chemical substances are volatile, or water soluble, enough may be absorbed into the medium to inhibit the growth of the fungi used. This is a test for fungistatic activity, however, and is not a test for fungicidal properties and, therefore, should not be used for testing fungicides.

Smyth and Smyth,[19] in their study on the fungicidal activity of pine oil, made use of a new method of test which possesses more possibilities than any of those previously suggested. The test organisms, fungi causing skin diseases, are grown on Sabouraud's agar and bits of the fungus growth 1 mm. square are removed from the agar slant and are used as the test culture. After exposing these small squares of the matted growth to the action of the fungicide for specified time periods, they are removed, rinsed in 95 per cent alcohol to remove the excess fungicide, and then washed in saline to remove the excess alcohol. The squares are then transferred to Sabouraud's agar and incubated at 20° C. for fourteen days. The time periods of test range from five to thirty minutes. This method is far more practical than others suggested for this purpose, and in addition eliminates the possibility of fungistatic activity. The time periods of test also are reasonable and logical.

Probably the most objectionable feature of this method is the difficulty of cutting the fungus growth into pieces 1 mm. square and then removing the agar from the bits of growth. There is no need for using such small pieces of culture, and also it is unnecessary to remove the agar. Instead of rinsing in 95 per cent alcohol, it is sufficient to simply rinse out the fungicide in water, but using a time period sufficient to remove all of it. This would hardly be accomplished in the ten-second time period specified in the method. Furthermore, it is not necessary to incubate cultures of the fungi employed for one to two months before using as test organisms. A much shorter time period is sufficient. Because of the very small amount of culture used in this method, the test is not sufficiently severe to offer the usual margin of safety.

In spite of previous work in this field there still seems to be a need for a suitable laboratory method for testing fungicides. The methods already employed do not simulate practical conditions, do not test for penetration, have not been sufficiently checked by clinical tests, and are not sufficiently severe. For certain other technical reasons the methods proposed in the past do not seem applicable for testing fungicides intended for the treatment of epidermophytosis.

EXPERIMENTAL

We have recently had occasion to make a laboratory and clinical study of fungicides which are recommended for the treatment of epidermophytosis or "athlete's foot." Since the laboratory methods of tests available did not seem practical enough to indicate whether or not the fungicides tested would be effective under clinical conditions of use, we found it necessary to develop a new severe test which more nearly approaches clinical conditions met with in practice. The test, as finally perfected, was then checked by extensive clinical tests. It was found that those fungicides which pass this test within the minimum time period of the test are effective in the treatment of epidermophytosis and that those which do not pass the test in the maximum time period are not clinically effective.

Fungicides recommended for treating this condition must of necessity kill or inhibit the fungi causing it. In the past most investigators in this field have made use of more than one test organism and usually efforts were made to use those fungi which are actually involved in epidermophytosis. This is not only desirable but necessary, since there is a certain amount of specificity in the fungicidal activity of many preparations used for this purpose. Somewhat typical of the test organisms employed are those used by Gould and Carter,[20] namely, *Epidermophyton interdigitale*, *Epidermophyton purpureum*, and *Epidermophyton gypseum*. These three fungi are the ones most commonly associated with ringworm of the feet and toes, or so-called "athlete's foot."

In our studies five such test organisms were employed. The following fungi were used: *Epidermophyton interdigitale*, *Trichophyton purpureum*, *Trichophyton gypseum*, *Epidermophyton inguinale*, and *Trichophyton rosaceum*. All of these organisms are not present in every case of epidermophytosis and they are not always present in pure culture. They do cause this condition in pure or mixed cultures, and fungicides must kill all of them if they are to constitute an effective general treatment for this condition.

FUNGICIDAL TEST

The laboratory method for testing for fungicidal activity employed by us simulates practical conditions more closely than those previously described and suggested for this purpose. This method is as follows:

> Using the test organisms listed above, each organism is streaked over the entire surface of Sabouraud's agar (20 c.c.) in a 9 cm. Petri dish, using a dry sterile cotton swab inoculated with cultures of these organisms which had been grown on Sabouraud's agar for five days at room temperature. These plates are incubated at room temperature for five days. The agar cultures are then cut into 1 cm. squares, or into disks by means of a cork borer, 1 cm. in diameter. The fungicide to be tested is poured over the surface of the cultures so as to entirely flood the plates, which requires 15 c.c. or more for each plate. After five, fifteen, and thirty minutes, one of the squares or disks of culture and agar from each plate is removed and placed into 10 c.c. of sterile broth. The excess fungicide is washed out of the matted culture by shaking the broth tube lightly for five minutes. At the end of this time

the block of culture is removed from the broth and spread with the culture side down over the surface of a sterile slant of Sabouraud's agar. These slants are then incubated at room temperature for three weeks and observed for growth. An effective fungicide should kill these test organisms within five minutes.

This test is simple, requires very little material, makes use of those organisms which are the most common cause of epidermophytosis, makes use of a solid medium in which these organisms grow most luxuriantly, uses a large inoculum, and avoids satisfactorily any bacteriostatic effect of the fungicides tested. The method is practical, as has been proved by clinical tests, and has been found to be satisfactory for the purpose of indicating which fungicides will be effective under practical conditions of use and which will not.

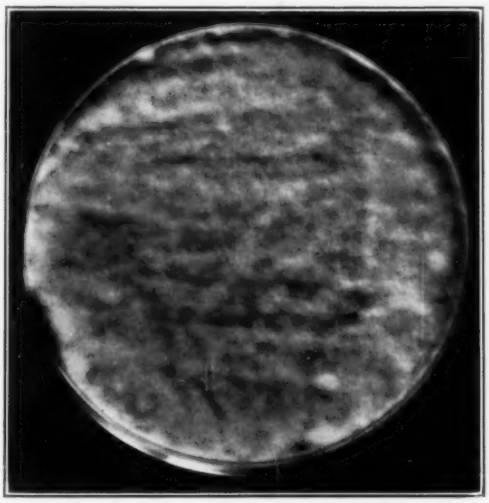


Fig. 1.—Plate showing even distribution of one of the test organisms (*E. interdigitale*) when applied with cotton swab to plate of Sabouraud's agar.

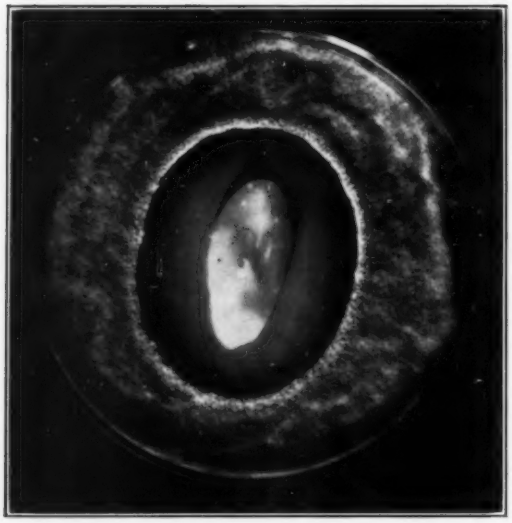
It was found that those preparations which kill these test organisms under the conditions of this test within five minutes are clinically effective in the treatment of this infection. Clinical tests on 30 patients suffering from "athlete's foot" showed that such fungicides are effective in clinical practice. Clinical tests on 30 other patients also showed that a preparation which did not pass this test —that is, did not kill these test organisms—in thirty minutes, was ineffective in the treatment of epidermophytosis.

It is apparent that this laboratory method of test is satisfactory for the purpose of designating which fungicides will and which will not be effective in the treatment of epidermophytosis. It is also evident that this method is more practical, more simple, and that it more nearly simulates conditions as they exist in practice than the other methods which have been suggested. Comparative tests now in progress also indicate that concordant results are readily obtained with this method by different workers. It is for these reasons that the above method is suggested as suitable for testing fungicides recommended for use in the treatment of epidermophytosis.

FUNGISTATIC TESTS

In the fungistatic tests used in the past the chemical substances tested were simply incorporated into the media used, either dextrose broth or Sabouraud's agar, into which the test organisms were then inoculated, incubated, and observed for inhibition of growth. While this method is helpful in showing which

a.



b.

Fig. 2.—Ointment A on (*a*) Sabouraud's agar and (*b*) on Sabouraud's agar containing 10 per cent blood serum, showing the slight difference in zone of penetration and fungistatic activity of this ointment in the presence and absence of blood serum.

substances will inhibit the growth of fungi and which will not, it offers no means of measuring penetration of the fungistatic substance. *Bacteriostatic* activity of antiseptics is tested by means of the serum-agar plate method or the agar cup-

plate method as described by Reddish[21] and U. S. Department of Agriculture Circular No. 198.[22] Under these conditions test is made for penetration as well as inhibition of growth. Similar technique may be employed in testing for *fungistatic* activity of various kinds of preparations which are applied for long periods of time.

Ointments, salves, powders, etc., which are used in treating epidermophytosis may be tested for fungistatic activity as follows: Add 2 c.c. of sterile normal horse serum to 18 c.c. of Sabouraud's agar, which has been melted and cooled to 45° C., and pour into 9 cm. Petri dishes. After the agar has hardened, streak each plate with one of the test fungi, using a dry sterile cotton swab. The preparation being tested is then added to the surface of the agar. Ointments and salves are melted and streaked on the surface of the inoculated agar by means of a glass rod, and powders are applied to the surface with a small spatula. These are applied to but a small portion of the plate, the ointment along a streak about 1.0 cm. wide and extending across the middle of the plate, and the powders applied to an area of about 1 cm. square. The plates are incubated at room temperature for five days. If the preparation tested is fungistatic, there will be a clear zone in the medium around the area where it was applied. The extent of this clear zone shows the amount of penetration and the effectiveness of the fungistatic ingredient.

Liquids and jellies which are applied for long time periods in treating epidermophytosis are tested by the agar cup-plate technique.[21, 22] The serum agar is prepared as for the agar plate method but when it is poured, a vial 1.0 cm. in diameter is placed in the center of the plate and left there until the agar has solidified, after which it is removed and the small cracks and crevices in the resulting cup are filled with two or three drops of melted agar. The entire surface of each plate, including the cup, is then inoculated with one of the above test organisms. After this has been done, 0.5 c.c. of the liquid or jelly being tested is placed into the cup, the plates covered with an unglazed clay top, and incubated at room temperature for five days. Penetration and fungistatic activity will be shown by a clear zone around the cup.

Although we have made no clinical tests with ointments, salves, powders, etc., which show penetration and fungistatic activity by these tests, it can fairly be assumed that such preparations will be beneficial under practical conditions of use. In the first place the agar plate and the agar cup-plate tests employed are almost identical with the standard Food and Drug Administration methods used for testing for inhibitory power of similar antiseptic preparations, the only essential differences being the use of Sabouraud's agar and the five selected fungi for test organisms. The test is severe since it requires activity in the presence of blood serum, penetration through a medium simulating body tissue to some degree, and definite inhibition of the test organisms to a measurable distance from the site of application of the preparations tested. For these reasons we consider these tests for fungistatic activity sufficiently practical to be interpreted in terms of effectiveness under clinical conditions of use. Those preparations which penetrate this medium and exert fungistatic effect on the test organisms used will be of some effectiveness under clinical conditions of use.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

SULFANILAMIDE, Experimental Study of Behavior of, Adair, F. L., Hesseltine, H. C., and Hac, L. R. J. A. M. A. **111**: 766, 1938.

Sulfanilamide has been found in the cervical secretion and menstrual fluid, but in amounts so small that its bactericidal action on the gonococcus is questionable.

The criterion of cure of gonorrhea should be based, if possible, on cultural studies as well as smears.

Sulfanilamide is excreted in breast milk, both free and as the acetyl derivative. The milk level is considerably above the blood level, and the drug is excreted in the milk for some time after the blood level is negligibly low. With doses of 2 and 4 gm. (30 and 60 grains) the total amount excreted was never greater than 1.5 per cent of the amount of the drug administered. It was still being excreted in small amounts seventy-two hours after medication had been discontinued.

Sulfanilamide is transmitted to the placenta and fetus of the rabbit and is associated with a marked increase in the mortality of the young. Sulfanilamide has also been found in the placenta and cord blood of the human being.

Until more is known of the tolerance of the human fetus and of the newborn for sulfanilamide, the drug should be administered only with the utmost caution during pregnancy and the period of lactation. If administered to the mother, breast feeding should be discontinued during the period that sulfanilamide is excreted in the milk.

PNEUMONIA, Use of Serum in the Treatment of the Higher Types of, Plummer, N. J. A. M. A. **111**: 694, 1938.

Pneumonia of the higher types is an important part of the pneumonia problem. In a collected series of 6,545 cases of pneumococcic pneumonia, over 50 per cent of the cases were of the higher types, 30 per cent being of types IV, V, VII, VIII, and XIV.

Antipneumococcus serum was used in 111 cases, with a rather marked clinical response and an appreciable effect on the mortality rate for the combined series of cases of pneumonia of types IV, V, VII, VIII, and XIV. At present there are available refined and concentrated preparations of horse and of rabbit serum that are high in antibody content and almost entirely free from reaction-causing substances. With such products the prospects are excellent for obtaining increasingly better results in the treatment of all types of pneumococcic pneumonia.

DIABETES MELLITUS, Size of Red Blood Corpuscle in, Mohr, C. F. Am. J. M. Sc. **196**: 67, 1938.

Studies of the red blood cell counts, hemoglobin, and hematocrit determinations have been made in 42 cases of uncomplicated diabetes mellitus, 18 cases of diabetic acidosis, and 4 cases of diabetic dwarfism.

The mean corpuscular volume was found to be 95 c. mm., or above, in 14.3 per cent of the uncomplicated diabetic and in 35 per cent of the cases of diabetic acidosis. This increase in corpuscular volume of the red blood cells could not be attributed to the acidosis per se.

ACETONE: Stable Sodium Nitroprusside Solution for Acetone Bodies in Urine, Ingham, J. Brit. M. J., Aug. 13, p. 348, 1938.

The reagents for the improved test are:

Solution No. 1.—Ammonium sulfate solution:

200 c.c. saturated solution of ammonium sulfate
200 c.c. ammonium hydroxide, sp. gr. 0.88

Solution No. 2.—Sodium nitroprusside solution:

10 gm. sodium nitroprusside crystals dissolved in 90 c.c. distilled water, and 1 c.c. concentrated nitric acid then added.

The Test: To 5 c.c. of urine in a test tube, add 5 c.c. of solution No. 1 and mix. Then add 1 c.c. of solution No. 2, shake, and allow to stand for a minute before judging the color reaction. A deep purple color will then have appeared if 0.1 per cent or more of acetone bodies is present. If only a very faint trace is present, the color will be merely deep red.

The saturated solution of ammonium sulfate used in solution No. 1 should be prepared as follows: Place 780 gm. of ammonium sulfate in a 2 liter flask, and add 1 liter of boiling distilled water. Shake immediately until dissolved, then allow to cool to room temperature. Decant the supernatant solution (or filter if necessary).

CALCULI: Blood Calcium, Phosphorus and Phosphatase in Urinary Lithiasis, Griffin, M., Osterberg, A. E., and Braasch, W. F. J. A. M. A. **111**: 683, 1938.

In cases of urinary lithiasis the values for blood calcium, phosphorus, and phosphatase exhibit no common change which can be termed characteristic of the group. Minor variations in the value for blood phosphorus, particularly a lowered value, are not unusual, but the concentration of blood calcium is very constant.

Patients with a high value for blood calcium or phosphatase who have urinary calculi should undergo a thorough investigation for some other coexistent pathologic condition.

In this study, hyperparathyroidism was found to be an etiologic factor in less than 0.2 per cent of the 1,206 cases of urinary lithiasis.

PNEUMONIA, Atypical, An Acute Infection of the Respiratory Tract With, Reimann, H. A. J. A. M. A. **111**: 2377, 1938.

In a series of 8 cases of an unusual, uniform, severe infection of the respiratory tract the disease was not caused by the virus of epidemic influenza or psittacosis, nor was it like other commonly described diseases.

The author was, therefore, led to regard it as a separate disease entity, pending the outcome of further experimental studies. The infection occurred in adults and began as a mild infection of the respiratory tract; this was followed by severe, diffuse, atypical pneumonia, and in 2 cases by the symptoms of encephalitis. Dyspnea, cyanosis, hoarseness, cough without sputum, drowsiness, and profuse sweating were the chief characteristics. The disease lasted several weeks. A filtrable infectious agent recovered from the nasopharynx of one patient and from the blood of another may have been etiologically related to the infection, but the evidence is incomplete. Experiments to clarify this point are under way.

PLACENTA, Pathology of, Bartholomew, R. A. J. A. M. A. **111**: 2276, 1938.

Infarction of the placenta is the most important pathologic condition to which this organ is subject, not only from the standpoint of frequency, but also because of its relationship to toxemia of pregnancy and maternal and fetal mortality.

The predisposing causes of placental infarction are, first, the hypercholesteremia of pregnancy, which favors the accumulation of lipoid cells at points of stress or injury of the placental arteries, similar to that seen with coronary occlusion, and, secondly, the trauma of fetal movements, which not only tends to cause deposits of lipoid cells at points of injury to the placental arteries, but also may break the endothelium over such a deposit or actually bring about rupture of the vessel, thus causing rapid thrombosis.

Toxemia of pregnancy probably results from the absorption of poisonous protein-split products of placental autolysis.

The poisonous products of placental autolysis are probably peptone, histamine, and guanidine. The pathologic effects of these poisons satisfactorily explain the symptoms and pathologic changes of toxemia of pregnancy.

The peculiar eclamptogenic character of placental autolysate is probably explained by the higher content of arginine in placental tissue, from which guanidine may be obtained.

Toxemia of pregnancy is associated with definite types of acute infarction of the placenta. In examining "unknown" placentas it is possible to diagnose pre-eclampsia, eclampsia, or abruptio placentae in 90 per cent of the cases.

BLOOD CHEMISTRY, Postmortem Determinations, Hamilton, R. C. Arch. Path. 26: 1135, 1938.

The concentrations of sugar, nonprotein nitrogen, and chlorides in blood obtained post mortem vary greatly, and their determination is of little value.

Creatinine is the only stable chemical constituent of post-mortem blood.

The creatinine content of blood obtained post mortem is no indication of the amount of renal damage present.

A post-mortem creatinine determination cannot be regarded as an index of renal function during life.

Chemical analysis of blood obtained post mortem is recommended because of its value in a few cases.

ANEMIA, Familial Hemolytic, Dimensions of Red Cells in, Hill, J. M. J. A. M. A. 111: 2179, 1938.

Microcytosis was found on at least one occasion in 6 of 7 cases of familial hemolytic anemia, while spherocytosis was found in 5 cases.

Spherocytosis may be absent in the presence of complicating factors, such as cirrhosis of the liver.

Macrospherocytosis was observed in one patient during crisis.

No significant correlation could be established between spherocytosis and the severity of the disease, onset of crisis, rapidity of regeneration of the red blood cells, or effect of splenectomy.

PREGNANCY TEST, The Xenopus (S. A. Clawed Toad), Elkan, E. R. Brit. M. J. 2: 1253, 1938.

The xenopus test allows a diagnosis of early pregnancy to be made within less than twenty-four hours.

No animals need be killed to obtain the result.

The reliability of the test does not seem to differ from that of the Aschheim-Zondek or the Friedman reaction.

The technique of this test is comparatively simple and very suitable for experimental work on the anterior-pituitary-like hormone of pregnancy.

For the test, 2 c.c. of untreated urine or 1 c.c. of extract is injected into the lymph sac under the dorsal skin of as many female toads as one cares to use. Some observers inject into the leg or into the peritoneal cavity. The toads are slippery and difficult to hold; the easiest way to deal with them is to hold them in a coarse meshed net and to inject through the meshes. There is no immediate reaction to ordinary urine. If extract is used which still contains a trace of alcohol or acetone, the toads react by secreting mucus from the skin glands in the neighborhood of the injection. The question of the "toxicity" of the urine seems to arise very rarely.

After the injection has been given, the toads are put into test jars where they sit on perforated platforms, so that they have no chance of eating their own spawn.

During the test the jars are kept at a temperature of 26° C. The shortest time observed between injection and oviposition was four hours and fifty minutes; the longest, twelve hours.

The eggs, little round balls, half black, half white, and of about 1 mm. in diameter, are covered with a sticky gelatinous substance. Normally these animals do not lay their eggs in bulk but distribute them over a wide area, sticking them on to water weeds one by one. In the test jar the eggs either stick to the underside of the platform or fall to the bottom of the jar. Their number varies enormously. Anything from five to six eggs upwards can be counted as a positive reaction. Tests in which only one or two eggs have been laid by one or two toads should be repeated. They will usually be found to be negative. In the absence of males these eggs are, of course, unfertilized. It should be emphasized that spontaneous ovulation in this species does not take place in captivity or under laboratory conditions. Females, even if kept under the best possible conditions, will never ovulate, except in the presence of a male.

TYPHOID FEVER, Agglutination in the Diagnosis of, and the Typhoid Carrier Condition, Bhatnager, S. S. Brit. M. J. 2: 1195, 1938.

A strain of *Bact. typhosum* which gives rise to pure Vi agglutination has been identified.

With the help of this strain the Vi antibody is shown to be produced in every case of typhoid fever, the inoculated individuals producing a higher Vi titer than the uninoculated.

Vi agglutination is shown to be a more reliable method of diagnosing typhoid fever in the inoculated than the time-honored O and H types of agglutination.

A correlation between the typhoid carrier condition and the presence of Vi antibody in the serum is described. The employment of Vi agglutination in the routine carrier examination and in tracing the source of typhoid infection is suggested.

SULFANILAMIDE, Studies on the Mechanism of the Action of, Lockwood, J. S., Coburn, A. F., and Stokinger, H. E. J. A. M. A. 111: 2259, 1938.

The effectiveness of sulfanilamide therapy is related to the type of lesion.

The function of the microorganism, which is strikingly depressed by sulfanilamide, is its capacity to invade tissue.

The effect of the drug on bacterial invasiveness seems to be influenced by the amount of debris present in the lesion.

These phenomena are being investigated experimentally to determine the mode of action of sulfanilamide.

TUBERCLE BACILLI, Spontaneous Lysis of, on Artificial Culture Media, Steenken, Wm., Jr. Am. Rev. Tuberc. 38: 777, 1938.

It has been shown that ripe cultures of human strains of the Rv variants undergo liquefaction on gentian violet egg and plain egg media, giving rise to a secondary, resistant growth of a different colony and bacillary morphology. The microorganisms from the liquefied area did not produce tuberculosis in the guinea pigs as did the original mother colony. Neither would they grow on any of the media mentioned. However, the secondary resistant type colony, although it did not produce progressive tuberculosis, grew on all of the laboratory media mentioned.

It was observed that the resistant colonies produced skin hypersensitivity in guinea pigs when inoculated intraperitoneally, intratesticularly, and subcutaneously, and that this might persist for one year or longer. When the guinea pigs were killed at the end of a year, autopsies showed no macroscopical lesions, except occasionally at the site of

injection of the intratesticularly infected animals, and these lesions were generally accompanied by a small amount of fibrosis without any demonstrable acid-fast microorganisms. Early lesions at times were caseous, and acid-fast bodies of marked pleomorphisms could be demonstrated.

The above lytic phenomenon is not only inherent in the H37 Rv culture, but has been shown to exist with other Rv variants of human origin. Also all of the cultures used in this experiment were of human origin, as proved by the present methods of differentiation.

It has been demonstrated that there is a definite lytic principle in the filtrate of the culture which acts upon the living bacilli within a definite range of pH.

It has also been observed that the bacillary morphology of the resistant colony is markedly different from the Rv variant.

ANEMIAS, Hemolyic, Hemolysins as the Cause of Clinical and Experimental, Dameshek, W. D., and Schwartz, S. O. Am. J. M. Sc. **196**: 769, 1938.

Isohemolysins of the immune-body type were discovered in the serum of 3 cases of acute hemolytic anemia.

Anti-guinea pig hemolytic serum was prepared by the injection of guinea pig red blood cells into rabbits. This serum possessed all the immunologic properties of the serum found in the clinical cases.

Hemolysis of the red blood cells of the guinea pig in vivo followed the injection of this serum.

By varying the dosage of anti-guinea pig hemolytic serum, various types of hemolytic syndromes were produced: fulminating hemolytic anemia with hemoglobinuria, acute hemolytic anemia, and subacute hemolytic anemia.

Various types of blood pictures could be reproduced at will: microspherocytosis, increased erythrocyte fragility, reticulocytosis, "pseudomacrocytic" blood picture, and so on.

The spherocyte is a small thick red blood cell, unaltered in volume though small in diameter, and unusually fragile to hypotonic salt solutions. Our observations point to the conclusion that spherocytosis is due to the activity of hemolysin and not to an abnormal anatomic peculiarity or to a disturbed formation of cells in the bone marrow. Since increased fragility is a function of the increased thickness of the red blood cell, it is dependent upon the same cause.

The authors believe that hemolytic syndromes are due to hemolysins, possibly of different types and present in different amounts, functioning slowly in some cases and violently in others. The various blood pictures of the hemolytic anemias, viz., anemia, spherocytosis, increased fragility, and reticulocytosis, are in all probability due to the effects of the varying activity of hemolysins, and modified by the individual's power to react.

Since the experimentally produced hemolytic syndromes and the numerous clinical types are closely comparable, the chief differences in the clinical syndromes may be a matter of the amount of functioning hemolysin present.

BLOOD SEDIMENTATION, Microsedimentation in Infants and in Children, Rogatz, J. L. Am. J. Dis. Child. **56**: 1037, 1938.

The Smith micrometric method was compared with the accepted Cutler macrometric method in 100 tests, performed during a period of fourteen months by different workers on 75 infants and older children of varying ages and with different illnesses. On the basis of the results it is concluded that the micrometric method, which requires minimal amounts of capillary blood, obtainable by pricking the finger, is a satisfactory test, is easy to use, and is completely reliable in its accuracy. One need not hesitate to replace existing macrometric methods with this test.

REVIEW

Books and Monographs for Review should be sent direct to the Editor,
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

Synopsis of Clinical Laboratory Methods*

THIS is an excellent guide for the graduate clinical technician as well as a text for the student. It covers all phases of clinical laboratory procedure, presenting an unusual wealth of material and choice of the most authoritative and recent methods. Dr. Bray's style of composition is at once direct and thorough, and his subject matter is extremely well organized, clear to the reader, and exhaustive.

In addition to regular methods the book includes special sections on the technique of basal metabolism tests, allergy tests, qualitative and quantitative methods for identification of poisons and foreign substances, surgical pathology, normal tables, preparation and use of indicators, and preparation of stains and reagents.

To the second edition have been added the many advances in clinical laboratory methods made during the past two years.

Among the additional procedures in this edition are serum phosphatase determination, titration of staphylococcus antitoxin in the blood serum, Ivy bleeding time, peroxidase-Giemsa staining method, cough plate method for diagnosis of whooping cough, the halo or diffraction method for measuring erythrocytes, determination of cyanates in the blood, 'phthalein elimination curve, determination of sulfanilamide in the blood, the differential heterophile agglutination for infectious mononucleosis, and determination of vitamin C.

*Synopsis of Clinical Laboratory Methods. By W. E. Bray, B.A., M.D., Professor of Clinical Pathology, University of Virginia; Director of Clinical Laboratories, University of Virginia Hospital. Cloth, ed. 2, 324 pages, 51 illustrations, 17 color plates, $4.50. The C. V. Mosby Co., St. Louis, Mo.